

Dean L. Engelhardt, et al.

Serial No.: 08/479,997

Filed: June 7, 1995

Page 60 [Reply Under 37 C.F.R. §1.111 (In Response To The
November 26, 2001 Office Action) - May 28, 2002]

REMARKS

Claims 454-567 were previously pending in this application. Those claims have been canceled in favor of new claims 576-825. Accordingly, claims 576-825 are presented for further examination on the merits.

Applicants appreciate the indication that prosecution has been reopened for their application and that the amendments submitted in their August 20, 2001 Appeal Brief have been entered. Applicants also appreciate that previous objections and rejections not reiterated in the November 26, 2001 Office Action have been withdrawn. Finally, Applicants acknowledge with thanks the courtesy that was extended to Dr. Dean L. Engelhardt and their attorney during the December 27, 2001 interview attended by Examiner Spiegler and Dr. Kenneth Horlick.

New Claims

Claims 576-825 are based upon Applicants' former and now canceled claims 454-567 except that the scope of the new claims differs in three respects as follows. In claims 576-657, Applicants are claiming polynucleotidyl compositions in which a *non-polypeptide*, non-radioactive label moiety Sig is covalently attached directly or through a chemical linkage to the phosphate moiety of at least one modified nucleotide. Of claims 576-657, claims 576, 596, 617 and 637 are independent, corresponding to former claims 454, 482, 511 and 539, respectively (except for the insertion of "polypeptide" in the new claims). In claims 658-735, the non-radioactive label moiety Sig in Applicants' polynucleotidyl compositions comprises biotin, iminobiotin, an electron dense component, a magnetic component, a metal-containing component, a fluorescent component, a

Dean L. Engelhardt, et al.

Serial No.: 08/479,997

Filed: June 7, 1995

Page 61 [Reply Under 37 C.F.R. §1.111 (In Response To The
November 26, 2001 Office Action) – May 28, 2002]

chemiluminescent component, a chromogenic component or a combination of any of the foregoing. Of claims 658-735, claims 658, 677, 697 and 716 are independent, corresponding to former claims 454, 482, 511 and 539, respectively. Finally, in claims 736-813, Applicants are claiming polynucleotidyl compositions in which the non-radioactive label moiety Sig is covalently attached to the phosphate moiety of at least one modified nucleotide through a chemical linkage comprising a polypeptide or a protein. Claims 736, 755, 775 and 794 are independent, again corresponding to former claims 454, 482, 511 and 539, respectively.

To help track the other new dependent claims against the former, canceled claims 454-567, a claim list has been compiled and is attached as Exhibit 1 to this Reply Amendment.

Entry of new claims 576-825 is respectfully requested.

The Rejection Under 35 U.S.C. §112, First Paragraph

Claims 461, 489, 518 and 546 stand rejected under 35 U.S.C. §112, first paragraph, for containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. In the November 26, 2001 Office Action (page 2), the Examiner stated:

These claims include the recitation "wherein chemical linkage comprises or includes an olefinic bond at the delta-position relative to the point of attachment" which does not appear in the specification. This recitation is considered new matter.

The rejection for new matter is respectfully traversed.

It is believed that the basis for the new matter rejection has been obviated by the presentation of the new claims that recite the " α -position relative to the

Dean L. Engelhardt, et al.

Serial No.: 08/479,997

Filed: June 7, 1995

Page 62 [Reply Under 37 C.F.R. §1.111 (In Response To The
November 26, 2001 Office Action) – May 28, 2002]

point of attachment" of the olefinic bond. The previously recited " δ -position" has been omitted from the new claims altogether. The new recitation is properly supported by Applicants' original disclosure. See, for example, the specification, page 3, lines 2 & 3 from the bottom of the page; page 11, line 7 in the second paragraph; and originally filed claim 78.

In view of the new claims, Applicants respectfully request reconsideration and withdrawal of the new matter rejection.

The Rejection Under 35 U.S.C. §112, Second Paragraph

Claims 455, 483, 512 and 540 stand rejected under 35 U.S.C. §112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. In the Office Action (page 3), the Examiner stated:

Claims 455, 483, 512 and 540 are indefinite over the recitation of "self-signaling or self-indicating or self-detecting" because it is not clear what is meant by this recitation. (i.e. it is not clear as to how a Sig can be considered [to] be self-signaling or self-indicating or self-detecting). For example, a fluorescent compound needs a specific wavelength of light to excite the compound to fluoresce and optical detection system to detect emitted fluorescence, therefore, it is not clear as to how a Sig (for example, a fluorescent compound) could be self-signaling or self-indicating or self-detecting. In other words, it is not clear as to how a Sig can be considered self-signaling or self-indicating or self-detecting without the use of an additional element to aid in the signaling, indicating or detecting of the Sig.

The indefiniteness rejection is respectfully traversed.

With respect to the phrase "self-signaling or self-indicating or self-detecting" in various new claims (577, 597, 618, 638, 659, 678, 698, 717, 737, 756, 776 and 795), Applicants respectfully maintain that this language is altogether proper

Dean L. Engelhardt, et al.

Serial No.: 08/479,997

Filed: June 7, 1995

Page 63 [Reply Under 37 C.F.R. §1.111 (In Response To The
November 26, 2001 Office Action) – May 28, 2002]

and passes the statutory strictures for definiteness. In light of Applicants' specification and the knowledge in the art, it is believed that a reader skilled in the art would readily comprehend the meaning of a non-radioactive label moiety Sig that is or renders a nucleotide or oligo- or polynucleotide self-signaling or self-indicating or self-detecting. From the specification, it would have been understood that "self-signaling or self-indicating or self-detecting" label moieties in the context of non-radioactive labeled nucleotides and oligo- or polynucleotides containing them provide a means for direct detection, including fluorescence and chemiluminescence.

In further support of the claim language at hand, Applicants would like to draw attention to eight documents listed below, of which four are U.S. patents (Exhibits 1-4) and four are scientific publications (Exhibits 5-8). The four U.S. patents include

U.S. Patent No. 4,649,121 ("self-indicating" in claims 6 & 7) [Exhibit 2];

U.S. Patent No. 5,233,044 ("self-indicating" in claim 1) [Exhibit 3];

U.S. Patent No. 4,981,653 ("self-indicating" assay device in claims 1-8)

[Exhibit 4]; and

U.S. Patent No. 4,408,202 ("self-indicating" reagents in claims 1, 20 & 50)

[Exhibit 5].

The terminology "self-indicating" is also used and recognized in the literature, particularly with respect to substrates. In this regard, Applicants can to the following four scientific articles.

Atherton et al., "***Self-indicating*** Activated Esters for Use in Solid Phase Peptide Synthesis. Fluorenylmethoxycarbonylamino Acid Derivatives of 3-Hydroxy-4-oxodihydrobenzotriazine," Journal Chemical Society, Chemical Communications 0(24):1763-1765 (1986) [Exhibit 6];

Dean L. Engelhardt, et al.

Serial No.: 08/479,997

Filed: June 7, 1995

Page 64 [Reply Under 37 C.F.R. §1.111 (In Response To The
November 26, 2001 Office Action) – May 28, 2002]

Valcour et al., "Evaluation of a Kinetic Method for Prostatic Acid
Phosphatase with Use of *Self-Indicating* Substrate, 2,6-Dichloro-4-Nitrophenyl
Phosphate," Clinical Chemistry 35(6):939-945 (1989) [Exhibit 7];

Rocco, R. M., "Fluorometric Determination of Alkaline Phosphatase in Fluid
Dairy Products: Collaborative Study," J. Assoc. Off. Anal. Chem. 73(6):542-549
(1990) [Exhibit 8]:

The purpose of the present study was to collaboratively examine a
new fluorometric assay for ALP in dairy products (3). The method is
based on a fluorometric substrate called *Fluorophos*®, which, when
acted upon by ALP, is converted to a highly fluorescent product. This
fluorometric quantitative assay is the first dairy product ALP test that
permits continuous and direct measurement of the released reaction
product from a *self-indicating* substrate. . . .

[Rocco, page 542, right column, first full ¶, emphasis added]

Osawa et al., "Prostatic Acid Phosphatase Assay with *Self-Indicating*
Substrate 2,6-Dichloro-4-acetylphenyl Phosphate," Clinical Chemistry 41(2):200-
203 (1995) [Exhibit 9]:

We characterize six *self-indicating* substrates . . .

[Osawa et al., page 200, abstract; emphasis added]

Discussion

To overcome several disadvantages involved in conventional methods
for PAP activity (1-8), we have developed a new assay and described
its performance. DCAPP, a *self-indicating* synthetic substrate, has
played a key role.

[page 202, right column, first ¶; emphasis added]

In summary, for the measurement of PAP activity, our kinetic method
involving the *self-indicating* substrate DCAPP showed satisfactory
performance on automated analyzers. . .

[page 203, left column, last ¶; emphasis added]

Dean L. Engelhardt, et al.

Serial No.: 08/479,997

Filed: June 7, 1995

Page 65 [Reply Under 37 C.F.R. §1.111 (In Response To The
November 26, 2001 Office Action) – May 28, 2002]

In light of Applicants' specification and the usage in the art as evidenced by attached Exhibits 1-8, reconsideration and withdrawal of the indefiniteness rejection is respectfully requested.

The First Rejection Under 35 U.S.C. §102

Claims 454-461, 463-474, 476-489, 491-502, 504-518, 520-531, 533-546, 548-559 and 561-567 stand rejected under 35 U.S.C. §102(e) as being anticipated by Ward et al., U.S. Patent No. 4,711,955. In the Office Action (pages 4-5), the Examiner stated:

Ward teaches modified nucleotides and methods of using and preparing the same. Specifically, Ward teaches the production and use of nucleic acid probes comprising a general structure (see abstract),

"wherein B represents a purine, 7-deazapurine, or pyrimidine moiety covalently bonded to the C1'-position of the sugar moiety, provided that when B is purine or 7-deazapurine, it is attached at the N9-position of the purine or 7-deazapurine and when B is pyrimidine, it is attached at the N1-position, wherein A represents a moiety (i.e. Sig) consisting of at least three carbon atoms which is capable of forming a detectable complex with a polypeptide when the compound is incorporated into a double-stranded ribonucleic acid, deoxyribonucleic acid duplex, or DNA-RNA hybrid; wherein the dotted line represents a chemical linkage joining B and A, provided that if B is purine, the linkage is attached to the 8-position of the purine, if B is 7-deazapurine, the linkage is attached to the 7-position of the deazapurine, and if B is pyrimidine, the linkage is attached to the 5-position of the pyrimidine and wherein each of x, y and z represents (H-, HO-, etc. see abstract) either directly or when incorporated into oligo- and polynucleotides, provide probes which are widely useful." (see abstract).

It is noted that the claims of the instant invention are broadly drawn to oligo- or polydeoxynucleotides or polyribonucleotides, wherein the Sig is covalently attached to the PM (or x, y or Z) directly **or through a chemical linkage**. Ward teaches the covalent attachment of the Sig to the PM through a chemical linkage (i.e. through the

Dean L. Engelhardt, et al.

Serial No.: 08/479,997

Filed: June 7, 1995

Page 66 [Reply Under 37 C.F.R. §1.111 (In Response To The
November 26, 2001 Office Action) – May 28, 2002]

linkage of the sugar (SM) and the base (BASE) moieties – see abstract), therefore, Ward teaches the instant claims 454, 482, 510, 511, 539 and 567, and claims 457, 485, 514, and 542.

With respect to claims 455-56, 458, 463-474, 476-481, 483-484, 486, 491-502, 504-509, 512-513, 520-531, 533-538, 540-541, 543, 548-559, 561-566 the reference teaches that the probe (for general formula, see abstract) comprising the Sig can be a self-signalling moiety such as biotin, fluorescent dyes, electron-dense reagents (such as ferritin, colloidal gold, ferric oxide) or enzymes (such as peroxidase, alkaline phosphatase) (col. 18, 9-68).

With respect to claims 459-461, 487-489, 516-518, and 544-546, Ward teaches:

"the chemical linkages may include *any of the well known bonds including carbon-carbon single bonds, carbon-carbon double bonds, carbon-nitrogen single bonds, or carbon-oxygen single bonds.* However, it is generally preferred that *the chemical linkage include an olefinic bond at the alpha-position relative to B.* The presence of such an alpha-olefinic bond serves to hold the moiety A away from the base when the base is paired with another in the well known double helix configuration. This permits interaction with polypeptide to occur more readily, thereby facilitating complex formation. Moreover, single bonds with greater rotational freedom may not always hold the moiety sufficiently apart from the helix to permit recognition by and complex formation with polypeptide. *It is even more preferred that the chemical linkage group be derived from a primary amine, and have the structure -CH.sub2-NH-,* since such linkages are easily formed utilizing any of the well known amine modification reactions. *Examples of preferred linkages derived from allylamine and allyl-(3-amino-2-hydroxyl-1-propyl) either groups have the formulae -CH.dbd.CH-CH.sub2-NH-and STR12## respectively.*

Although these linkages are preferred, others can be used, including particularly olefin linkage arms with other modifiable functionalities, such as thiol, carboxylic acid, and epoxide functionalities.

This rejection could be overcome by amending the claims by deleting the recitation "*or through a chemical linkage*".

[emphasis in the November 26, 2001 Office Action]

The anticipation rejection is respectfully traversed.

Dean L. Engelhardt, et al.

Serial No.: 08/479,997

Filed: June 7, 1995

Page 67 [Reply Under 37 C.F.R. §1.111 (In Response To The
November 26, 2001 Office Action) - May 28, 2002]

In response, Applicants respectfully point out that the Ward '955 Patent limits the attachment of the non-radioactive labels to the non-disruptive base positions of the pyrimidine, purine or deazapurine, namely, the 5-, 8- and 7-positions, respectively. In contrast, the claims in the Engelhardt application are directed to compositions in which the non-polypeptide, non-radioactive label moiety Sig is attached to the phosphate moiety -- not even to the base, let alone to the aforementioned Ward positions (the 5-, 8- or 7-positions of a pyrimidine, purine or deazapurine, respectively). There are at least two significant reasons why it is incorrect and improper to characterize the specific base labeling positions in the Ward '955 Patent for attaching non-radioactive labels as being indirectly attached to the phosphate moiety through the linkage of the sugar.

First, the sugar is a distinct element of a nucleotide and it is not recognized in the art to my knowledge as being an indirect linkage of the phosphate moiety to the base moiety. A person of ordinary skill in the art would simply not consider the attachment to the base moiety in a nucleotide to be an indirect linkage to the phosphate moiety in a nucleotide.

Second, it should not be overlooked that the three elements making up a nucleotide (sugar, phosphate and base) are not only different structurally, but they are different chemically, such that these elements are subject to *different* chemical reactions. Again, a person of ordinary skill in the art would simply not treat the sugar, phosphate and base moieties in a nucleotide as interchangeable elements.

In light of the foregoing remarks, Applicants respectfully request reconsideration and withdrawal of the first rejection for anticipation.

Dean L. Engelhardt, et al.

Serial No.: 08/479,997

Filed: June 7, 1995

Page 68 [Reply Under 37 C.F.R. §1.111 (In Response To The
November 26, 2001 Office Action) - May 28, 2002]

The Second Rejection Under 35 U.S.C. §102

Claims 454-459, 463-464, 472-475, 478-487, 491-492, 500-503, and 506-510 stand rejected under 35 U.S.C. §102(b) as being anticipated by or, in the alternative, under 35 U.S.C. §103(a) as obvious over Halloran et al. (J. of Immun. (1966), 96(3):373-378). In the Office Action (pages 5-7), the Examiner stated:

Halloran teaches a Sig label (i.e. a protein) attached to nucleic acids (pg. 373, 374 - Fig. 1 and col. 2). Specifically, Halloran teaches the preparation of nucleotide protein conjugates through the covalent linkage of a protein to a nucleotide (on the phosphate moiety) with a carbodiimide coupling agent (Fig. 1). Halloran also teaches that this conjugation can be applied to mononucleotides, oligonucleotides, and DNA (pg. 373, col. 1). Therefore, Halloran teaches an oligonucleotide, comprising at least one modified nucleotide having the formula Sig-PM-SM-BASE, wherein the Sig is covalently attached to the PM directly, said Sig being a moiety capable of non-radioactive detection (i.e. instant claims 454, 481, 482, 509, and 510).

With respect to claims 455 and 483, Halloran teaches that the Sig is a protein, which is or renders the nucleotide self-signaling or self-indicating or self-detecting. Halloran teaches the detection of the protein and DNA through Amidoschwartz and Feulgan staining, respectively (pg. 374, col. 2).

With respect to claims 456, 458, 464, 472-475, 478, 479, 484, 486, 492, 500-503, and 506-508 Halloran teaches the addition of proteins (such as HSA and poly-lysine) (pg. 375, Table 1), which comprise at least three carbon atoms.

With respect to claims 457, 459, 463, 480, 485, 487, 491, and 508 Halloran teaches the covalent attachment of -P-O-, said chemical linkage of -CH₂NH-, and where the Sig is covalently attached to the PM through a phosphorus atom or phosphate oxygen (pg. 374, Fig. 1).

Therefore, even though Halloran does not teach the hybridization of an oligodeoxynucleotide to a nucleic acid of interest, or a portion thereof, but it is an inherent property of an oligonucleotide to hybridize to a nucleic acid of interest (or portion thereof), which is complementary to the oligonucleotide.

It is noted that in In re Best (195 USPQ 430) and In re Fitzgerald (205 USPQ 594) discuss the support of rejections wherein

Dean L. Engelhardt, et al.

Serial No.: 08/479,997

Filed: June 7, 1995

Page 69 [Reply Under 37 C.F.R. §1.111 (In Response To The
November 26, 2001 Office Action) - May 28, 2002]

the prior art discloses subject matter which there is reason to believe inherently includes functions that are newly cited or is identical to a product instantly claimed. In such a situation the burden is shifted to the applicants to "prove that subject matter shown to be in the prior art does not possess characteristics relied on" (205 USPQ 594, second column, first full paragraph).

In the alternative, it would have been obvious to one of ordinary skill in the art at the time the invention was made to have used the oligonucleotide preparation method of Halloran of conjugating a protein to an oligonucleotide (through the PM), in order to have produced a compound that was complementary to a nucleic acid of interest for detection and identification purposes. If the hybridization property of oligonucleotides is not inherent, the disclosure of oligonucleotides, per se, suggests hybridizability, which is a well known characteristic. Therefore, the burden is shifted to the applicant to prove this otherwise.

The second anticipation rejection is respectfully traversed.

In response, Applicants are pleased to present the Declaration of Dr. Charles W. Parker, who is Professor *Emeritus* of Medicine, Department of Microbiology and Immunology at Washington University School of Medicine in St. Louis, Missouri. Dr. Parker is well recognized as an investigator and author in immunology. Two of Dr. Parker's 1966 papers are cited in the November 26, 2001 Office Action and they serve as the basis for eight of the prior art rejections. As indicated in his Declaration which is attached as Exhibit A, Dr. Parker has spent over five decades in the field. His work has involved conjugate chemistry and the use of conjugated products, including radiolabeled proteins and nucleotide-protein conjugates, for immunization and radioimmunoassays. Dr. Parker's distinguished career is described on the first five or six pages in his Declaration (Exhibit A). As indicated in his Declaration (Section 12, page 13), Dr. Parker is at least a person of ordinary skill in the art to which the present invention pertains.

Dean L. Engelhardt, et al.

Serial No.: 08/479,997

Filed: June 7, 1995

Page 70 [Reply Under 37 C.F.R. §1.111 (In Response To The
November 26, 2001 Office Action) – May 28, 2002]

In response to the second rejection, Applicants direct attention to and incorporate Dr. Parker's statements in his Declaration beginning on page 19, last paragraph, and continuing through most of page 22. A reading of the background to Dr. Parker's cited papers can also be found on page 14, last paragraph, continuing through the first half of page 19.

In view of the new claims and the statements in Dr. Parker's Declaration (Exhibit A), it is believed that the second rejection has been overcome. Reconsideration and withdrawal of the rejection is respectfully requested.

The Third Rejection Under 35 U.S.C. §102

Claims 511-516, 520-521, 529-532, 535, 536-544, 548, 549, 557-560, and 563-567 stand rejected under 35 U.S.C. §102(b) as being anticipated by or, in the alternative, under 35 U.S.C. §103(a) as obvious over Halloran et al. (J. of Immun. (1966), 96(3):379-385). In the Office Action (pages 7-9), the Examiner stated:

Halloran teaches the conjugation of proteins to mono, oligo, and polynucleotides (pg. 379, i.e. reference to preceding article – Halloran et al. (J. of Immun. (1966), 96(3):373-378), see teachings above. The teachings of Halloran (pgs. 373-378) are cited herein only to demonstrate content of Halloran (379-385)).

Halloran (pg. 381, column 2) teaches:

"The results of the immunologic studies indicate that nucleotides, oligonucleotides and DNA-protein conjugates can induce the formation of antibodies with nucleotide specificity. The antibodies react both with denatured DNA and with nucleotide protein conjugates. While immunologic response to analogous RNA protein preparations has not been studied, it may be presumed that antibodies to the different types of RNA could be obtained by the same procedure".

Therefore, Halloran teaches an oligoribonucleotide, comprising at least one modified nucleotide having the formula Sig-PM-SM-BASE, wherein the Sig is covalently attached to the PM

directly, said Sig being a moiety capable of non-radioactive detection (i.e. instant claims 511, 538, 539, 566, and 567).

With respect to claims 512 and 540, Halloran teaches that the Sig is a protein, which is or renders the nucleotide self-signaling or self-indicating or self-detecting. Halloran teaches the detection of the proteins and DNA through Amidoschwartz and Feulgan staining, respectively (pg. 374, col. 2).

With respect to claims 513, 515, 521, 529-532, 535-536, 541, 543, 549, 557-560, 563, and 564 Halloran teaches the addition of proteins (such as HSA and poly-lysine) (pg. 375, Table 1), which comprise at least three carbon atoms.

With respect to claims 514, 516, 520, 537, 542, 544, 548, and 565 Halloran teaches the covalent attachment of $-P-O-$, said chemical linkage of $-CH_2NH-$, and where the Sig is covalently attached to the PM through a phosphorus atom or phosphate oxygen (pg. 374, Fig. 1).

Therefore, even though Halloran does not teach the hybridization of an oligoribonucleotide to a nucleic acid of interest, or a portion thereof, but it is an inherent property of an oligoribonucleotide to hybridize to a nucleic acid of interest (or portion thereof), which is complementary to the oligonucleotide.

It is noted that in *In re Best* (195 USPQ 430) and *In re Fitzgerald* (205 USPQ 594) discuss the support of rejections wherein the prior art discloses subject matter which there is reason to believe inherently includes functions that are newly cited or is identical to a product instantly claimed. In such a situation the burden is shifted to the applicants to "prove that subject matter shown to be in the prior art does not possess characteristics relied on" (205 USPQ 594, second column, first full paragraph).

In the alternative, in view of the teachings of Halloran (pgs. 379-385) it would have been obvious to one of ordinary skill in the art at the time the invention was made to have modified the method of Halloran (pgs. 373-378) so as to have conjugated a protein to an RNA molecule in order to have achieved an equally effective compound for use in hybridization or antibody production. If the hybridization property of oligoribonucleotides is not inherent, the disclosure of oligoribonucleotides, per se, suggests hybridizability, which is a well known characteristic. Therefore, the burden is shifted to the applicant to prove this otherwise.

Dean L. Engelhardt, et al.

Serial No.: 08/479,997

Filed: June 7, 1995

Page 72 [Reply Under 37 C.F.R. §1.111 (In Response To The
November 26, 2001 Office Action) - May 28, 2002]

The third anticipation rejection is respectfully traversed.

Applicants respectfully direct attention to the Declaration of Dr. Charles W. Parker (Exhibit A). His statements found on page 23 and continuing through the first half of page 25 are incorporated here for Applicants' response to the third anticipation rejection.

In light of the new claims and the statements of Dr. Parker, Applicants respectfully request reconsideration and withdrawal of the third rejection.

The First Rejection Under 35 U.S.C. §103

Claims 462, 464, 469-471, 476, 477, 490, 492, 497-499, 504, and 505 stand rejected under 35 U.S.C. §103(a) as being unpatentable over Halloran et al. (J. of Immun. (1966), 96(3):373-378), as applied to claims 454-459, 463-464, 472-475, 478-487, 491-492, 500-503, and 506-510 above, and further in view of Falkow et al., U.S. Patent No. 4,358,535. In the Office Action (pages 9-11), the Examiner stated:

The teachings of Halloran are presented above. Specifically, Halloran teaches an oligonucleotide, comprising at least one modified nucleotide having the formula Sig-PM-SM-BASE, wherein the Sig is covalently attached to the PM directly, said Sig being a moiety capable of non-radioactive detection (i.e. protein). Halloran also describes specific linkages and attachments of the Sig and the PM. Halloran does not teach specific Sig's, such as fluorescent compounds, ligands, etc. of nucleic acid probes.

Falkow teaches methods and compositions for infectious disease diagnosis and epidemiology involving labeled nucleotide probes complementary to nucleic acid coding for a characteristic pathogen product (see abstract). Specifically, Falkow teaches:

"The probe may be RNA or DNA. The probe will normally have at least about 25 bases, more usually at least about 30 bases, and may have up to about 10,000 bases or more, usually having not more

than about 5,000 bases. The probe sequence will be at least substantially complementary to a gene coding for a product characteristic of the pathogen, usually a cytoplasmic product or released product, particularly an excreted product. The probe need not have perfect complementarity to the sequence to which it hybridizes; there may be 30% or more of mismatched pairs." (col. 2, ln. 42-54).

With respect to claims 462, 469, 490, and 497 Falkow teaches that enzymes of interest as labels (i.e. Sigs) include hydrolases, particularly esterases and glycosidases (i.e. which would be attached to the PM via a glycosidic linkage), or oxidoreductases, particularly peroxidases (col. 4, ln. 12-14).

With respect to claims 471 and 499, the reference teaches that fluorescent compounds include fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, etc. (col. 4, ln. 12-16).

With respect to claims 470 and 498, Falkow teaches that the probe can be labeled with heavy metals (i.e. which are catalytic) (col. 3, ln. 25-28).

With respect to claims 476-477 and 504-505, Falkow teaches:

"In some situations it may be feasible to employ an antibody (i.e. a polypeptide) which will bind specifically to the probe hybridized to the single stranded DNA of the pathogen. In this instance, the antibody would be labeled to allow for detection. The same types of labels which are used for the probe may also be bound to the antibody in accordance with known techniques." (col. 3, ln. 28-34).

"Other labels include ligands, which will serve as a specific binding member to a labeled antibody, fluorescers, chemiluminescers, enzymes, antibodies which can serve as a specific binding pair member for a labeled ligand, and the like. A wide variety of labels have been employed in immunoassays which can readily be employed in the present assay." (col. 3, ln. 38-45).

"Ligands and antiligands may be varied widely. Where a ligand has a natural receptor, namely ligands such as biotin, thyroxine, and cortisol, these ligands can be used in conjunction with the labeled naturally occurring receptors. Alternatively, any compound can be used, either haptenic or antigenic, in combination with an antibody." (col. 4, ln. 5-11).

Dean L. Engelhardt, et al.

Serial No.: 08/479,997

Filed: June 7, 1995

Page 74 [Reply Under 37 C.F.R. §1.111 (In Response To The
November 26, 2001 Office Action) – May 28, 2002]

In view of the teachings of Falkow, it would have been obvious to one of ordinary skill in the art at the time the invention was made to have modified the oligonucleotides of Halloran so as to have used alternative Sig labels, instead of using a protein, in order to have provided an equally effective compound for nucleic acid detection.

The first obviousness rejection is respectfully traversed.

Reference is made to the statements of Dr. Parker in his Declaration (Exhibit A), beginning on page 25, second paragraph, and continuing through the first half of page 27.

In view of the new claims and Dr. Parker's Declaration, Applicants respectfully request reconsideration and withdrawal of the first obviousness rejection.

The Second Rejection Under 35 U.S.C. §103

Claims 519, 521, 526-528, 533, 534, 547, 549, 554-556, 561, and 562 stand rejected under 35 U.S.C. §103(a) as being unpatentable over Halloran et al. (J. of Immun. (1966), 96(3):379-385), as applied to claims 511-516, 520-521, 529-532, 535, 536-544, 548, 549, 557-560, and 563-567 above, and further in view of Falkow et al., U.S. Patent No. 4,358,535. In the Office Action (pages 11-13), the Examiner stated:

The teachings of Halloran are presented above. Specifically, Halloran teaches an oligonucleotide, comprising at least one modified nucleotide having the formula Sig-PM-SM-BASE, wherein the Sig is covalently attached to the PM directly, said Sig being a moiety capable of non-radioactive detection (i.e. protein). Halloran also describes specific linkages and attachments of the Sig and the PM. Halloran does not teach specific Sig's, such as fluorescent compounds, ligands, etc. of nucleic acid probes.

Falkow teaches methods and compositions for infectious disease diagnosis and epidemiology involving labeled nucleotide probes

Dean L. Engelhardt, et al.

Serial No.: 08/479,997

Filed: June 7, 1995

Page 75 [Reply Under 37 C.F.R. §1.111 (In Response To The
November 26, 2001 Office Action) - May 28, 2002]

complementary to nucleic acid coding for a characteristic pathogen product (see abstract). Specifically, Falkow teaches:

"The probe may be RNA or DNA. The probe will normally have at least about 25 bases, more usually at least about 30 bases, and may have up to about 10,000 bases or more, usually having not more than about 5,000 bases. The probe sequence will be at least substantially complementary to a gene coding for a product characteristic of the pathogen, usually a cytoplasmic product or released product, particularly an excreted product. The probe need not have perfect complementarity to the sequence to which it hybridizes; there may be 30% or more of mismatched pairs." (col. 2, ln. 42-54).

With respect to claims 519, 526, 547, and 554 Falkow teaches that enzymes of interest as labels (i.e. Sigs) include hydrolases, particularly esterases and glycosidases (i.e. which would be attached to the PM via a glycosidic linkage), or oxidoreductases, particularly peroxidases (col. 4, ln. 12-14).

With respect to claims 528 and 556, the reference teaches that fluorescent compounds include fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, etc. (col. 4, ln. 12-16).

With respect to claims 527 and 555, Falkow teaches that the probe can be labeled with heavy metals (i.e. which are catalytic) (col. 3, ln. 25-28).

With respect to claims 533-534 and 561-562, Falkow teaches:

"In some situations it may be feasible to employ an antibody (i.e. a polypeptide) which will bind specifically to the probe hybridized to the single stranded DNA of the pathogen. In this instance, the antibody would be labeled to allow for detection. The same types of labels which are used for the probe may also be bound to the antibody in accordance with known techniques." (col. 3, ln. 28-34).

"Other labels include ligands, which will serve as a specific binding member to a labeled antibody, fluorescers, chemilumescers, enzymes, antibodies which can serve as a specific binding pair member for a labeled ligand, and the like. A wide variety of labels have been employed in immunoassays which can readily be employed in the present assay." (col. 3, ln. 38-45).

"Ligands and antiligands may be varied widely. Where a ligand has a natural receptor, namely ligands such as biotin, thyroxine, and

Dean L. Engelhardt, et al.

Serial No.: 08/479,997

Filed: June 7, 1995

Page 76 [Reply Under 37 C.F.R. §1.111 (In Response To The
November 26, 2001 Office Action) – May 28, 2002]

cortisol, these ligands can be used in conjunction with the labeled naturally occurring receptors. Alternatively, any compound can be used, either haptenic or antigenic, in combination with an antibody." (col. 4, ln. 5-11).

In view of the teachings of Falkow, it would have been obvious to one of ordinary skill in the art at the time the invention was made to have modified the oligonucleotides of Halloran so as to have used alternative Sig labels, instead of using a protein, in order to have provided an equally effective compound for nucleic acid detection.

The second obviousness rejection is respectfully traversed.

Applicants incorporate the statements of Dr. Parker from his Declaration (Exhibit A), beginning on page 27, last paragraph, and continuing through most of page 29.

In view of the new claims and Dr. Parker's statements, reconsideration and withdrawal of the second obviousness rejection is respectfully requested.

The Third Rejection Under 35 U.S.C. §103

Claims 460-461, 465-468, 488-489, and 493-496 stand rejected under 35 U.S.C. §103(a) as being unpatentable over Halloran et al. (J. of Immun. (1966), 96(3):373-378), as applied to claims 454-459, 463-464, 472-475, 478-487, 491-492, 500-503, and 506-510 above, and further in view of Ward et al., U.S. Patent No. 4,711,955. In the Office Action (pages 13-14), the Examiner stated:

The teachings of Halloran are presented above. Specifically, Halloran teaches an oligonucleotide, comprising at least one modified nucleotide having the formula Sig-PM-SM-BASE, wherein the Sig is covalently attached to the PM directly, said Sig being a moiety capable of non-radioactive detection (i.e. protein). Halloran also describes specific linkages and attachments of the Sig and the PM. Halloran does not teach the chemical linkages comprising an allylamine group or an ether group and Halloran does not teach a Sig comprising ferritin.

Dean L. Engelhardt, et al.

Serial No.: 08/479,997

Filed: June 7, 1995

Page 77 [Reply Under 37 C.F.R. §1.111 (In Response To The
November 26, 2001 Office Action) – May 28, 2002]

Ward teaches that the probe (for general formula, see abstract) comprising the Sig can be a self-signalling moiety such as biotin, fluorescent dyes, electron-dense reagents (such as ferritin, colloidal gold, ferric oxide), or enzymes (such as peroxidase, alkaline phosphatase) (col. 18, 9-68).

With respect to specific linkages, Ward teaches:

"the chemical linkages may include *any of the well known bonds including carbon-carbon single bonds, carbon-carbon double bonds, carbon-nitrogen single bonds, or carbon-oxygen single bonds.* However, it is generally preferred that *the chemical linkage include an olefinic bond at the alpha-position relative to B.* The presence of such an alpha-olefinic bond serves to hold the moiety A away from the base when the base is paired with another in the well known double helix configuration. This permits interaction with polypeptide to occur more readily, thereby facilitating complex formation. Moreover, single bonds with greater rotational freedom may not always hold the moiety sufficiently apart from the helix to permit recognition by and complex formation with polypeptide. *It is even more preferred that the chemical linkage group be derived from a primary amine, and have the structure -CH.sub2-NH-,* since such linkages are easily formed utilizing any of the well known amine modification reactions. *Examples of amino-2-hydroxyl-1-propyl ether groups have the formulae -CH.dbd.CH-CH.sub2-NH- and STR12## respectively.*

Although these linkages are preferred, others can be used, including particularly olefin linkage arms with other modifiable functionalities, such as thiol, carboxylic acid, and epoxide functionalities.

It is also noted that the instant specification teaches that the "Sig component of the nucleotides in accordance with this invention and the nucleotides and polynucleotides incorporating the nucleotides of this invention containing the Sig component are equivalent to and useful for the same purposes as the nucleotides described in U.S. patent application Serial No. 255,223, now U.S. Patent NO. 4,711,955". (pg. 97).

In view of the teachings of Ward, it would have been obvious to one of ordinary skill in the art at the time the invention was made to have modified the method of linkages of Halloran to include allylamine, ether, or any other well known chemical linkage, instead of carbodiimide linkage as taught by Halloran, so as to have achieved an

Dean L. Engelhardt, et al.

Serial No.: 08/479,997

Filed: June 7, 1995

Page 78 [Reply Under 37 C.F.R. §1.111 (In Response To The
November 26, 2001 Office Action) – May 28, 2002]

equally effective linkage between the Sig and the phosphate moiety. Furthermore, one would have been motivated to use the Sig labels of Ward (i.e. ferritin), instead of using a protein, in order to have provided an equally effective compound for nucleic acid detection.

The third obviousness rejection is respectfully traversed.

In response, Applicants incorporate the statements of Dr. Parker from his Declaration (Exhibit A), beginning at the bottom of page 29, and continuing through the first two lines on page 32.

In light of the new claims and Dr. Parker's statements, Applicants respectfully request reconsideration and withdrawal of the third obviousness rejection.

The Fourth Rejection Under 35 U.S.C. §103

Claims 517-518, 522-525, 545-546, and 550-553, stand rejected under 35 U.S.C. §103(a) as being unpatentable over Halloran et al. (J. of Immun. (1966), 96(3):379-385), as applied to claims 511-516, 520-521, 529-532, 535, 536-544, 548, 549, 557-650, and 563-567 above, and further in view of Ward et al., U.S. Patent No. 4,711,955. In the Office Action (pages 15-16), the Examiner stated:

The teachings of Halloran are presented above. Specifically, Halloran teaches an oligonucleotide, comprising at least one modified nucleotide having the formula Sig-PM-SM-BASE, wherein the Sig is covalently attached to the PM directly, said Sig being a moiety capable of non-radioactive detection (i.e. protein). Halloran also describes specific linkages and attachments of the Sig and the PM. Halloran does not teach the chemical linkages comprising an allylamine group or an ether group and Halloran does not teach a Sig comprising ferritin.

Ward teaches that the probe (for general formula, see abstract) comprising the Sig can be a self-signalling moiety such as biotin, fluorescent dyes, electron-dense reagents (such as ferritin, colloidal gold, ferric oxide), or enzymes (such as peroxidase, alkaline phosphatase) (col. 18, 9-68).

Dean L. Engelhardt, et al.

Serial No.: 08/479,997

Filed: June 7, 1995

Page 79 [Reply Under 37 C.F.R. §1.111 (In Response To The
November 26, 2001 Office Action) – May 28, 2002]

With respect to specific linkages, Ward teaches:
"the chemical linkages may include *any of the well known bonds including carbon-carbon single bonds, carbon-carbon double bonds, carbon-nitrogen single bonds, or carbon-oxygen single bonds.* However, it is generally preferred that *the chemical linkage include an olefinic bond at the alpha-position relative to B.* The presence of such an alpha-olefinic bond serves to hold the moiety A away from the base when the base is paired with another in the well known double helix configuration. This permits interaction with polypeptide to occur more readily, thereby facilitating complex formation. Moreover, single bonds with greater rotational freedom may not always hold the moiety sufficiently apart from the helix to permit recognition by and complex formation with polypeptide. *It is even more preferred that the chemical linkage group be derived from a primary amine, and have the structure -CH.sub2-NH-,* since such linkages are easily formed utilizing any of the well known amine modification reactions. *Examples of samino-2-hydroxyl-1-propyl) either groups have the formulae -CH.dbd.CH-CH.sub2-NH- and STR12## respectively.*

Although these linkages are preferred, others can be used, including particularly olefin linkage arms with other modifiable functionalities, such as thiol, carboxylic acid, and epoxide functionalities.

It is also noted that the instant specification teaches that the "Sig component of the nucleotides in accordance with this invention and the nucleotides and polynucleotides incorporating the nucleotides of this invention containing the Sig component are equivalent to and useful for the same purposes as the nucleotides described in U.S. patent application Serial No. 255,223, now U.S. Patent NO. 4,711,955". (pg. 97).

In view of the teachings of Ward, it would have been obvious to one of ordinary skill in the art at the time the invention was made to have modified the method of linkages of Halloran to include allylamine, ether, or any other well known chemical linkage, instead of carbodiimide linkage as taught by Halloran, so as to have achieved an equally effective linkage between the Sig and the phosphate moiety. Furthermore, one would have been motivated to use the Sig labels of Ward (i.e. ferritin), instead of using a protein, in order to have provided an equally effective compound for nucleic acid detection.

Dean L. Engelhardt, et al.

Serial No.: 08/479,997

Filed: June 7, 1995

Page 80 [Reply Under 37 C.F.R. §1.111 (In Response To The
November 26, 2001 Office Action) – May 28, 2002]

The fourth obviousness rejection is respectfully traversed.

In response, Applicants incorporate the statements of Dr. Parker from his Declaration (Exhibit A). Those statements begin near the top of page 32, and they continue through substantially most of page 33.

Applicants respectfully request reconsideration and withdrawal of the fourth obviousness rejection in light of the new claims and Dr. Parker's statements.

The Fifth Rejection Under 35 U.S.C. §103

Claims 475 and 503 stand rejected under 35 U.S.C. §103(a) as being unpatentable over Ward et al., U.S. Patent No. 4,711,955, as applied to claims 454-461, 463-474, 476-489, 491-502, 504-518, 520-531, 533-546, 548-559 and 561-567, above, and further in view of Halloran et al. (J. of Immun. (1966), 96(3):373-378). In the Office Action (pages 16-17), the Examiner stated:

The teachings of Ward are presented above. Specifically, Ward teaches the probe comprising Sig-PM-SM-BASE, wherein the covalent attachment of the Sig to the PM through a chemical linkage (i.e. through the linkage of the sugar (SM) and the base (BASE) moieties – see abstract). Ward also teaches that a variety of Sig labels can be used in detection (col. 18, ln. 21-28), but does not teach the Sig label comprising polylysine.

The teachings of Halloran are presented above. Specifically, Halloran teaches the addition of proteins (such as HSA and poly-lysine) to the PM, which can be used in detection with the Amidoschwartz staining procedure, for example (referenced to the pg. 375, Table 1 - referenced).

In view of the teachings of Halloran, it would have been obvious to one of ordinary skill in the art at the time the invention was made to have modified the compound of Ward to include to a Sig comprising polylysine, instead of a Sig (such as rhodamine), so as to have achieved an equally effective compound for nucleic acid detection.

The fifth obviousness rejection is respectfully traversed.

Dean L. Engelhardt, et al.

Serial No.: 08/479,997

Filed: June 7, 1995

Page 81 [Reply Under 37 C.F.R. §1.111 (In Response To The
November 26, 2001 Office Action) - May 28, 2002]

In response, Applicants incorporate the statements of Dr. Parker from his Declaration (Exhibit A). His statements begin with the last paragraph on page 33, and they continue through the first line on page 36.

Reconsideration and withdrawal of the fifth obviousness rejection is respectfully requested, particularly in view of the new claims and Dr. Parker's statements.

The Sixth Rejection Under 35 U.S.C. §103

Claims 532 and 560 stand rejected under 35 U.S.C. §103(a) as being unpatentable over Ward et al., U.S. Patent No. 4,711,955, as applied to claims 454-461, 463-474, 476-489, 491-502, 504-518, 520-531, 533-546, 548-559 and 561-567 above, and further in view of (J. of Immun. (1966), 96(3):379-385). In the Office Action (pages 17-18), the Examiner stated:

The teachings of Ward are presented above. Specifically, Ward teaches the probe comprising Sig-PM-SM-BASE, wherein the covalent attachment of the Sig to the PM through a chemical linkage (i.e. through the linkage of the sugar (SM) and the base (BASE) moieties - see abstract). Ward also teaches that a variety of Sig labels can be used in detection (col. 18, ln. 21-28), but does not teach the Sig label comprising polylysine.

The teachings of Halloran are presented above. Specifically, Halloran teaches the addition of proteins (such as HSA and poly-lysine) to the PM of a oligoribonucleotide, which can be used in detection with the Amidoshwartz staining procedure, for example (pg. 379, i.e. reference to preceding article - Halloran et al. (J. of Immun. (1966), 96(3):373-378).

In view of the teachings of Halloran, it would have been obvious to one of ordinary skill in the art at the time the invention was made to have modified the compound of Ward to include to a Sig comprising polylysine, instead of a Sig (such as rhodamine), so as to have achieved an equally effective compound for nucleic acid detection.

Dean L. Engelhardt, et al.

Serial No.: 08/479,997

Filed: June 7, 1995

Page 82 [Reply Under 37 C.F.R. §1.111 (In Response To The
November 26, 2001 Office Action) – May 28, 2002]

The sixth obviousness rejection is respectfully traversed.

In response, Applicants incorporate the statements of Dr. Parker in his Declaration (Exhibit A). His statements begin at the top of page 36 and they continue through the first three lines on page 37.

Reconsideration and withdrawal of the sixth obviousness rejection is respectfully requested, particularly in light of the new claims and Dr. Parker's statements on the matter.

The Seventh Rejection Under 35 U.S.C. §103

Claims 462, 490, 519, and 547 stand rejected under 35 U.S.C. §103(a) as being unpatentable over Ward et al., U.S. Patent No. 4,711,955, as applied to claims 454-461, 463-474, 476-489, 491-502, 504-518, 520-531, 533-546, 548-559 and 561-567 above, and further in view of Falkow et al., U.S. Patent No. 4,358,535. In the Office Action (pages 18-19), the Examiner stated:

The teachings of Ward are presented above. Specifically, Ward teaches the probe comprising Sig-PM-SM-BASE, wherein the covalent attachment of the Sig to the PM through a chemical linkage (i.e. through the linkage of the sugar (SM) and the base (BASE) moieties – see abstract). Ward also teaches that a variety of Sig labels (such as peroxidase and alkaline phosphatase) can be used in detection (col. 18, ln. 24-28), but does not teach the Sig label comprising a glycosidic linkage (i.e. using a Sig comprising a glycosidase).

The teachings of Falkow are presented above. Specifically, Falkow teaches enzymes of interest as labels (i.e. Sigs) include hydrolases, particularly esterases and glycosidases (i.e. which would be attached to PM via a glycosidic linkage), or oxidoreductases, particularly peroxidases (col. 4, ln. 12-14).

In view of the teachings of Falkow, it would have been obvious to one of ordinary skill in the art at the time the invention was made to have modified the compound of Ward to include to a Sig comprising a glycosidic linkage (i.e. a Sig comprising a glycosidase), instead of a Sig

Dean L. Engelhardt, et al.

Serial No.: 08/479,997

Filed: June 7, 1995

Page 83 [Reply Under 37 C.F.R. §1.111 (In Response To The
November 26, 2001 Office Action) – May 28, 2002]

(such as peroxidase, utilizing a different linkage), so as to have achieved an equally effective compound for nucleic acid detection.

The seventh obviousness rejection is respectfully traversed.

It is respectfully submitted that it would not have been obvious to one of ordinary skill in the art to have modified Ward's compound to include a glycosidic linkage instead of peroxidase, in view of Falkow's '535 Patent and its disclosure of enzymes as labels. One would simply not have arrived at the Engelhardt invention from a combined reading of the Ward and Falkow patents. Ward's Patent does not disclose among its compounds a non-polypeptide, non-radioactive label moiety Sig attached to the nucleotidyl phosphate moiety in an oligo- or polynucleotide, as generally set forth in the Engelhardt claims. Furthermore, other Engelhardt claims recite a non-polypeptide, non-radioactive label moiety Sig, or the members of Sig (biotin, iminobiotin, an electron dense component, a magnetic component, a metal-containing component, a fluorescent component, a chemiluminescent component, a chromogenic component, a hapten or a combination of any of the foregoing), or that Sig is covalently attached to the phosphate moiety through a chemical linkage comprising a polypeptide or a protein. The element Sig in the Engelhardt claims is not an enzyme as disclosed in the Falkow '535 Patent. Thus, the addition of the Falkow '535 Patent does not provide the necessary disclosure which would have motivated or allowed a person of ordinary skill in the art to arrive at the claims in the Engelhardt application from a combined reading of both the Ward and Falkow patents.

In view of the new claims and foregoing remarks, Applicants respectfully request reconsideration and withdrawal of the seventh obviousness rejection.

Dean L. Engelhardt, et al.

Serial No.: 08/479,997

Filed: June 7, 1995

Page 84 [Reply Under 37 C.F.R. §1.111 (In Response To The
November 26, 2001 Office Action) – May 28, 2002]

Information Disclosure Statement

Recently, some 29 documents came to the attention of Applicants' undersigned attorney. These documents came to light earlier this month from a third party. As part of their duty of disclosure and candor, Applicants are submitting these documents in their Third Supplementary IDS attached as Exhibit B.

Favorable action is respectfully urged.

* * * * *

Dean L. Engelhardt, et al.

Serial No.: 08/479,997

Filed: June 7, 1995

Page 85 [Reply Under 37 C.F.R. §1.111 (In Response To The
November 26, 2001 Office Action) – May 28, 2002]

SUMMARY AND CONCLUSIONS

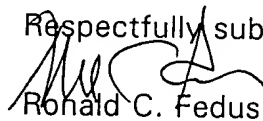
Claims 576-825 have been added in place of the former claims which have been canceled.

The fee for adding new claims 576-825 is \$1652, based upon the presentation of 74 additional claims [74 claims X \$18 = \$1332], and four new independent claims [4 claims X \$80 = \$320]. Authorization for these claim fees is set forth in the accompanying Transmittal. This Reply is also accompanied by a Request For Extension Of Time (3 months) and authorization for the fee therefor.

No fee or fees are believed due for filing this Reply. In the event that any other fee or fees are due, however, authorization is hereby given to charge the amount of any such fee(s) to Deposit Account No. 05-1135, or to credit any overpayment thereto.

If a telephone conversation would further the prosecution of the present application, Applicants' undersigned attorney request that he be contacted at the number provided below.

Respectfully submitted,



Ronald C. Fedus

Registration No. 32,567

Attorney for Applicants

ENZO LIFE SCIENCES, INC.
(formerly Enzo Diagnostics, Inc.)
c/o ENZO BIOCHEM, INC.
527 Madison Avenue, 9th Floor
New York, New York 10022
Telephone: (212) 583-0100
Facsimile: (212) 583-0150

EL839968769US

Dean L. Engelhardt et al.
U.S. Patent Application Serial No. 08/479,997
Exhibit 1 to May 28, 2002 Reply Under 37 C.F.R. §1.111

<u>New Claim(s)</u>	<u>Subject Matter (abbrev.)</u>	<u>Corresponding Former Claims (Now Canceled)</u>
576	independent claim	454
577	self-signaling or self-indicating or self-detecting	455
578	Sig comprises 3 carbons	456
579	covalent attachment	457
580	chemical linkage does not interfere with ability of Sig	458
581	chemical linkage members	459
582	chemical linkage -- allylamine	460
583	olefinic bond at α -position	461
584	chemical linkage . . . glycosidic	462
585	PM = mono-, di- or tri-phosphate	463
586	Markush members of Sig	464
587	electron dense component . . . ferritin	465
588	magnetic component . . . magnetic oxide	467
589	magnetic oxide . . . ferric oxide	468
590	metal containing component . . . catalytic	470
591	Markush members . . . fluorescent	471
592	Sig moiety attached terminal . . .	478
593	sugar moiety has H atom at 2'	479
594	sugar moiety has O atom at 2' & 3'	480
595	comprises at least one ribonucleotide	481
596	independent	482
597	same as 577	
598	same as 578	
599	same as 579	
600	same as 580	
601	same as 581	
602	same as 582	
603	same as 583	
604	same as 584	
605	x and y . . . mono-, di & tri-phosphate	491
606	same as 586	

Dean L. Engelhardt et al.

Serial No. 08/479,997

Filed: June 7, 1995

Page 2 [Exhibit 1 to May 28, 2002 Reply Under 37 C.F.R. §1.111]

<u>New Claim(s)</u>	<u>Subject Matter (abbrev.)</u>	<u>Corresponding Former Claims (Now Canceled)</u>
607	same as 587	
608	same as 588	
609	same as 589	
610	same as 590	
611	same as 591	
612	same as 592	
613	same as 593	
614	both y and z of terminal nucleotide comprises O atom at 2' & 3'	508
615	same as 595	
616	structural formula for oligo- or poly	510
617	independent	511
618	same as 577	
619	same as 578	
620	same as 579	
621	same as 580	
622	same as 581	
623	same as 582	
624	same as 583	
625	same as 584	
626	same as 585	
627	same as 586	
628	same as 587	
629	same as 588	
630	same as 589	
631	same as 590	
632	same as 591	
633	same as 592	
634	same as 593	
635	same as 594	
636	same as 595	
637	independent	539
638	same as 577	
639	same as 578	
640	same as 579	
641	same as 580	

Dean L. Engelhardt et al.

Serial No. 08/479,997

Filed: June 7, 1995

Page 3 [Exhibit 1 to May 28, 2002 Reply Under 37 C.F.R. §1.111]

<u>New Claim(s)</u>	<u>Subject Matter (abbrev.)</u>	<u>Corresponding Former Claims (Now Canceled)</u>
642	same as 581	
643	same as 582	
644	same as 583	
645	same as 584	
646	same as 605	
647	same as 586	
648	same as 587	
649	same as 588	
650	same as 589	
651	same as 590	
652	same as 591	
653	same as 592	
654	same as 593	
655	same as 614	
656	same as 595	
657	same as 616	
658	independent	
659	same as 577	454
660	same as 578	
661	same as 579	
662	same as 580	
663	same as 581	
664	same as 582	
665	same as 583	
666	same as 584	
667	same as 585	
668	same as 587	
669	same as 588	
670	same as 589	
671	same as 590	
672	same as 591	
673	same as 592	
674	same as 593	
675	same as 594	
676	same as 595	
677	independent482

Dean L. Engelhardt et al.

Serial No. 08/479,997

Filed: June 7, 1995

Page 4 [Exhibit 1 to May 28, 2002 Reply Under 37 C.F.R. §1.111]

<u>New Claim(s)</u>	<u>Subject Matter (abbrev.)</u>	<u>Corresponding Former Claims (Now Canceled)</u>
678	same as 577	
679	same as 578	
680	same as 579	
681	same as 580	
682	same as 581	
683	same as 582	
684	same as 583	
685	same as 584	
686	same as 605	
687	same as 587	
688	same as 588	
689	same as 589	
690	same as 590	
691	same as 591	
692	same as 592	
693	z of said terminal nucleotide comprises H atom at 2'	507
694	same as 614	
695	same as 615	
696	same as 616	
697	independent	
698	same as 577	511
699	same as 578	
700	same as 579	
701	same as 580	
702	same as 581	
703	same as 582	
704	same as 583	
705	same as 584	
706	same as 585	
707	same as 587	
708	same as 588	
709	same as 589	
710	same as 590	
711	same as 591	
712	same as 592	

Dean L. Engelhardt et al.

Serial No. 08/479,997

Filed: June 7, 1995

Page 5 [Exhibit 1 to May 28, 2002 Reply Under 37 C.F.R. §1.111]

<u>New Claim(s)</u>	<u>Subject Matter (abbrev.)</u>	<u>Corresponding Former Claims (Now Canceled)</u>
713	same as 593	
714	same as 594	
715	same as 595	
716	independent	
717	same as 577	539
718	same as 578	
719	same as 579	
720	same as 580	
721	same as 581	
722	same as 582	
723	same as 583	
724	same as 584	
725	same as 605	
726	same as 587	
727	same as 588	
728	same as 589	
729	same as 590	
730	same as 591	
731	same as 592	
732	same as 693	
733	same as 614	
734	comprising at least one deoxyribonucleotide	538
735	same as 616	
736	independent	454
737	same as 577	
738	same as 578	
739	same as 579	
740	same as 580	
741	same as 585	
742	same as 586	
743	same as 587	
744	same as 588	
745	same as 589	
746	same as 590	
747	same as 591	

Dean L. Engelhardt et al.

Serial No. 08/479,997

Filed: June 7, 1995

Page 6 [Exhibit 1 to May 28, 2002 Reply Under 37 C.F.R. §1.111]

<u>New Claim(s)</u>	<u>Subject Matter (abbrev.)</u>	<u>Corresponding Former Claims (Now Canceled)</u>
748	terminally ligated	558
749	comprises polylysine	560
750	avidin, streptavidin and anti-hapten Ig	561
751	almost same as 592	
752	same as 593	
753	same as 594	
754	same as 595	
755	independent	482
756	same as 577	
757	same as 578	
758	same as 579	
759	same as 580	
760	same as 605	
761	same as 586	
762	same as 587	
763	same as 588	
764	same as 589	
765	same as 590	
766	same as 591	
767	same as 748	558
768	same as 749	560
769	same as 750	561
770	Sig attached via polypeptide or protein chemical linkage to terminal nucleotide	478
771	same as 693	
772	same as 614	
773	comprising at least one ribonucleotide	509
774	structural formula	510
775	independent	511
776	same as 577	
777	same as 578	
778	same as 579	
779	same as 580	
780	PM = mono-, di- or tri-phosphate and Sig is covalently attached	520
781	same as 586	

Dean L. Engelhardt et al.

Serial No. 08/479,997

Filed: June 7, 1995

Page 7 [Exhibit 1 to May 28, 2002 Reply Under 37 C.F.R. §1.111]

<u>New Claim(s)</u>	<u>Subject Matter (abbrev.)</u>	<u>Corresponding Former Claims (Now Canceled)</u>
782	same as 587	
783	same as 588	
784	same as 589	
785	same as 590	
786	same as 591	
787	same as 748	
788	same as 749	
789	same as 750	
790	almost same as 592	
791	same as 593	
792	same as 594	
793	same as 595	
794	independent	
795	same as 577	539
796	same as 578	
797	same as 579	
798	almost same as 580	
799	same as 605	
800	same as 586	
801	same as 587	
802	same as 588	
803	same as 589	
804	same as 590	
805	same as 591	
806	same as 748	
807	same as 749	
808	same as 750	
809	almost same as 592	
810	same as 693	
811	same as 614	
812	comprising at least one deoxyribonucleotide	566
813	structural formula	567
814	similar to 770	
815	same as 749	
816	same as 750	
817	see claim 814	

presented here is made up by two nearly trigonal planar co-ordinated gold atoms. The two planes in each molecule are connected by P-C-P bridges with Au-P distances lying between 2.344(7) and 2.384(7) Å. The P-Au-P angles are slightly distorted from the ideal 120°.

The environment of the gold atoms is displayed in Figure 1. The geometry is close to ideal trigonal planar with P-Au-P angles ranging from 114.7 to 124.9° for Au(1), from 117.7 to 121.5° for Au(2), from 117.6 to 122.4° for Au(1') and from 115.7 to 127.3° for Au(2'). The Au(1) atom is 0.034 Å out of the least-squares plane through the P atoms, the Au(2') atom is 0.021 Å out of the plane, whereas the other two gold atoms are situated nearly within the least-squares plane of the P atoms. Such deviations from the ideal 120° angle and small deviations from planarity are also observed in other trigonal planar co-ordinated gold complexes.⁹ In each molecule two of these trigonal planar co-ordinated gold atoms are connected via P-C-P bridges. The planes in molecule 1 [gold atoms Au(1) and Au(2)] are tilted by a dihedral angle of 15.9°, whereas the corresponding planes of the other molecule are nearly parallel with a dihedral angle of 1.1°. As a consequence of this connection, short Au...Au contacts are observed [3.040(1) Å for Au(1)-Au(2) and 3.050(1) Å for Au(1')-Au(2')]. Such short contacts are frequently observed in Au^I derivatives, even when the Au atoms are in separate molecules.^{10,13}

The Au-P distances are situated in the range from 2.344(7) to 2.384(7) Å (average: 2.358 Å) and are in the same region as in many other Au-P bond-containing gold complexes.^{9-12,14} It should be noted that for the more usual two-co-ordination the Au-P bond length is markedly shorter (typically ca. 2.25 Å). The solvent molecules (toluene) are situated in interstices between the gold-clusters.

Financial support provided by the Swiss National Science Foundation is gratefully acknowledged.

Received, 7th July 1986; Com. 928

References

- 1 N. C. Baenziger, K. M. Dittmore, and J. R. Doyle, *Inorg. Chem.*, 1974, 13, 805.
- 2 L. J. Guggenberger, *J. Organomet. Chem.*, 1974, 81, 271.
- 3 C. B. Colburn, W. E. Hill, C. A. McAuliffe, and R. V. Parish, *J. Chem. Soc., Chem. Commun.*, 1979, 218.
- 4 W. Ludwig and W. Meyer, *Helv. Chim. Acta*, 1983, 65, 934.
- 5 Lj. Manojlovic-Muir and K. W. Muir, *J. Chem. Soc., Chem. Commun.*, 1982, 1155, and references therein.
- 6 J. Kozelka, H. R. Oswald, and E. Dubler, *Acta Crystallogr., Sect. C*, 1986, 42, 1002.
- 7 G. M. Sheldrick, SHELXS-84, Crystal Structure Solution, University of Göttingen, 1984.
- 8 G. M. Sheldrick, SHELX-76, Program for Crystal Structure Determination, University of Cambridge, 1976.
- 9 J. A. Muir, M. M. Muir, and S. Arias, *Acta Crystallogr., Sect. B*, 1982, 38, 1318.
- 10 P. G. Jones, G. M. Sheldrick, R. Uson, and A. Laguna, *Acta Crystallogr., Sect. B*, 1980, 36, 1486.
- 11 J. A. Muir, M. M. Muir, and S. Arias, *Acta Crystallogr., Sect. B*, 1982, 38, 2047.
- 12 H. Schmidbauer, A. Wohlleben, F. Wagner, O. Orama, and G. Hüttner, *Chem. Ber.*, 1977, 110, 1748.
- 13 V. Jiang, S. Alvarez, and R. Hoffmann, *Inorg. Chem.*, 1985, 24, 749.
- 14 P. G. Jones, *Gold Bull.*, 1981, 14, 102; 1981, 14, 159; 1983, 16, 114; 1986, 19, 46.

Self-indicating Activated Esters for Use in Solid Phase Peptide Synthesis. Fluorenylmethoxycarbonylamino Acid Derivatives of 3-Hydroxy-4-oxodihydrobenzotriazine

Eric Atherton, Linda Cameron, Morten Meldal, and Robert C. Sheppard
MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, U.K.

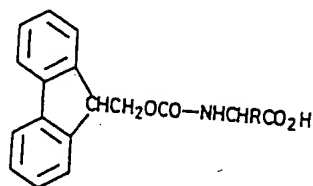
The title esters are effective acylating agents in solid phase peptide synthesis; completion of acylation is indicated by fading of the transient yellow colour produced by ionisation of the liberated hydroxy component.

We have reported¹ that pentafluorophenyl esters² of fluorenylmethoxycarbonyl (Fmoc) amino acids (1) are efficient acylating agents in solid phase peptide synthesis under polar polyamide conditions.³ Their use notably simplifies the conduct of solid phase synthesis by avoiding individual preactivation procedures, and provides a particularly simple solution to the problem of automatic peptide synthesiser design. However, the additional u.v. absorption introduced by the aryl ester and the liberated phenol makes quantitative spectroscopic monitoring⁴ of the acylation step more difficult. This is especially the case when catalyst 1-hydroxybenzotriazole is added to the reaction mixture¹ to enhance further the reactivity of the pentafluorophenyl ester derivatives. We report now a new series of Fmoc-amino acid activated esters which are efficient in peptide synthesis and which offer an entirely new opportunity for non-destructive

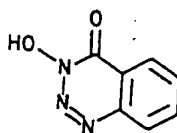
qualitative and quantitative monitoring of acylation reactions under continuous flow^{4,5} conditions.

The favourable acylating properties of esters of 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine, (HODhbt) (2) were recognized by König and Geiger in 1970,⁶ but no substantial application in solid phase synthesis has apparently been reported. We find that these esters of Fmoc-amino acids are very easily prepared and are generally stable crystalline solids,[†] most of which may be stored at low temperature for long periods without significant decomposition. Fmoc-Ile-ODhbt reacted with glycyl-polydimethylacrylamide resin⁴ in dimethylformamide (DMF) at a rate closely similar to that of

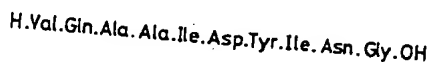
[†] Details of melting points etc. may be obtained from us prior to full publication.



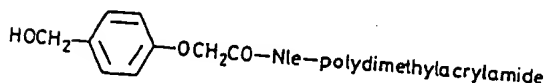
(1)



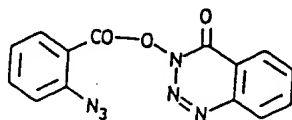
(2)



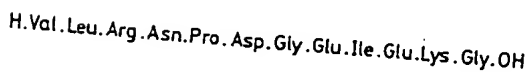
(3)



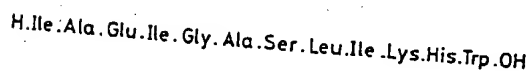
(4)



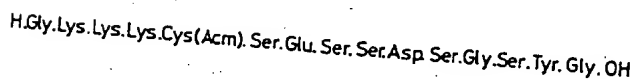
(5)



(6)



(7)



(8)

Acm = S-acetamidomethyl

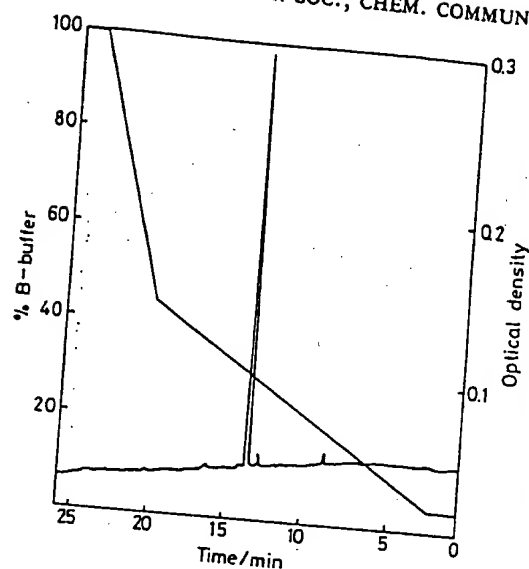


Figure 1. Analytical h.p.l.c. of total crude decapeptide on Aquapori RP-300. Reservoir A contained 0.1% aq. trifluoroacetic acid; E contained 90% acetonitrile, 10% A. After 2 min elution with 5% B, a linear gradient of 5–45% B was developed over 18 min and then 45–100% over 5 min; flow rate 1.5 ml/min. The effluent was monitored at 230 nm.

dimethylaminopyridine (DMAP) catalyst.[‡] All peptide bond-forming reactions utilised the appropriate Fmoc-amino acid Dhbt ester (4 equiv.) in DMF. Urea was added to the reaction mixture[§] for incorporation of the final valine residue (see below). Fmoc groups were cleaved by 20% piperidine-DMF.

In this first experiment, the progress of the synthesis was followed by observing persistence of the initial yellow colouration of the column, although for safety acylation times were tentatively set considerably longer. The following very approximate times (in min, unless otherwise indicated) were noted[§] for fading of the resin to its original off-white state with actual total reaction times in parentheses: Asn-Gly, 15 (35); Ile-Asn, 30 (65); Tyr-Ile, 18 (60); Asp-Tyr, 10 (40); Ile-Asp, 15 (40); Ala-Ile, 10 (40); Ala-Ala, 10 (40); Gln-Ala, 30 (130); and Val-Gln, 20 h (24 h). The exceptionally long reaction time noted for the final valine residue is in agreement with previous evidence for strong association of the peptide chains within the resin matrix was provided by slower release of dibenzofulvene-piperidine adduct during deprotection steps.^{4,9}

The completed decapeptide was cleaved from the resin with 95% aqueous trifluoroacetic acid; detachment was 92%

the corresponding symmetrical anhydride and about five times more rapidly than the uncatalysed pentafluorophenyl ester. During these and other early reactions, we observed that a transient bright yellow colour appeared on the resin during the acylation reaction, although the solution remained colourless in the absence of dissolved base. When acylation was complete the resin returned close to its initial off-white shade. We attribute this yellow colour to ionisation of liberated hydroxy component (2) by resin bound amino groups. Thus the Fmoc-amino acid activated ester provides both an effective acylating agent and a sensitive indicator of the presence of residual unreacted amino groups.

As before,¹ the efficiency of these new derivatives in solid phase peptide synthesis was tested by preparation of the difficult acyl carrier protein decapeptide sequence (3). The continuous flow variant^{4,5} of the Fmoc-polyamide procedure was used. The polydimethylacrylamide resin was supported in rigid, macroporous kieselguhr particles^{4,7} and was functionalised with a norleucine internal reference amino acid and the acid labile linkage agent as in (4). Esterification of the C-terminal Fmoc-glycine residue utilised the pentafluorophenyl ester derivative in the presence of 4-*N,N*-

[‡] Remarkably, the oxodihydrobenzotriazine ester appears to be relatively ineffective in ester-forming reactions, even in the presence of DMAP catalyst. The pentafluorophenyl ester provides a convenient alternative to symmetrical anhydrides previously used for this step. With Fmoc-glycine pentafluorophenyl ester (5 equiv.) in the presence of DMAP (1 equiv.), esterification is complete in 1–2 h.

[§] More recently, we have constructed a sensitive and accurate photometric system for monitoring resin-colour. The results obtained are generally consistent with visual estimation, except that both isoleucine residues now give approximately equal (36, 40 min) times for complete decolouration. With the more precise measurements now possible, we routinely allow a much shorter (10 min) safety factor after acylation is indicated as complete.

complete as judged by the glycine:norleucine analysis of residual resin. Unpurified decapeptide had amino acid analysis; Gly, 1.00; Asp, 1.91; Ile, 1.80; Tyr, 0.91; Ala, 1.87; Glu, 0.96; Val, 0.94. After h.p.l.c. purification (Figure 1; for conditions see ref. 1), the amino acid analysis was Gly, 1.00; Asp, 1.96; Ile, 1.92; Tyr, 0.95; Ala, 2.04; Glu, 0.98; Val, 0.98. A latter synthesis of the same sequence gave satisfactory results using only 2 equiv. of Dhbt ester except for the final valine (4 equiv., no urea). Satisfactory syntheses have also been achieved of the peptide sequences (6)–(8).

During the preparative work, we confirmed the observations of König and Geiger⁶ that esters of (2) prepared with the aid of dicyclohexylcarbodiimide (DCCI) may be accompanied by the by-product (5). Traces of (5) may easily be detected by h.p.l.c. [e.g. Fmoc-Gly-ODhbt and (5) emerge at 26.8 and 24.3 min respectively on Aquapore 300 using a gradient of 0–100% B (see caption Figure 1) in 40 min]. All Fmoc-amino acid derivatives should therefore be rigorously purified before use in solid phase synthesis since the azidobenzoate (5) is an effective chain terminating agent. In some early experiments, traces of contaminating azidobenzoyl peptides were detected. Formation of (5) is minimised by preparation of the active esters in a non-polar solvent (tetrahydrofuran) rather than in polar DMF, although the latter is to be preferred for the less soluble, side chain reactive amino acids, asparagine and glutamine. Almost complete suppression of (5) is obtained by preformation of the Fmoc-amino acid–DCCI adduct 4 min before addition of (2).

We conclude that Fmoc-amino acid Dhbt esters are valuable alternatives to other acylating species previously employed in solid phase synthesis. They are easily prepared, generally crystalline and apparently stable to storage,[†] yet are exceptionally reactive towards nitrogen nucleophiles. Their

[†] The long-term stability of these esters is not yet known. We recommend storage at low temperature (–20°C), especially for the derivatives of asparagine, glutamine, and arginine.

very favourable racemisation-resistant properties were established by König and Geiger.⁶ They provide a unique opportunity for continuous, non-invasive monitoring of solid phase synthesis^{||} with potential for automation. We shall report shortly on the design and operation of a fully automated peptide synthesiser utilising Dhbt esters in which acylation times are established individually for each coupling reaction as the synthesis proceeds.

We are grateful to Mr R. Cotton (ICI Pharmaceuticals Division) who first suggested to us that these esters might be useful alternatives to pentafluorophenyl derivatives in solid phase synthesis.

Received, 4th August 1986; Com. 1110

References

- 1 E. Atherton and R. C. Sheppard, *J. Chem. Soc., Chem. Commun.*, 1985, 165; E. Atherton, A. Dryland, P. Goddard, L. Cameron, J. D. Richards, and R. C. Sheppard, *Proc. 9th Am. Pept. Symp.*, Toronto, 1985, Pierce Chemical Co., Rockford 1985, p. 249.
- 2 L. Kisfaludy and I. Schön, *Synthesis*, 1983, 325.
- 3 R. Arshady, E. Atherton, D. L. J. Clive, and R. C. Sheppard, *J. Chem. Soc., Perkin Trans. 1*, 1981, 529.
- 4 A. Dryland and R. C. Sheppard, *J. Chem. Soc., Perkin Trans. 1*, 1986, 125.
- 5 R. C. Sheppard, *Chem. Br.*, 1983, 19, 402.
- 6 W. König and R. Geiger, *Chem. Ber.*, 1970, 103, 2034.
- 7 E. Atherton, E. Brown, R. C. Sheppard, and A. Rosevear, *J. Chem. Soc., Chem. Commun.*, 1981, 1151.
- 8 F. Westall and A. B. Robinson, *J. Org. Chem.*, 1970, 35, 2842.
- 9 E. Atherton, A. Dryland, R. C. Sheppard, and J. D. Wade, *Proc. 8th Am. Pept. Symp.*, Tucson 1983, Pierce Chemical Co., Rockford 1983, p. 45.

^{||} Patent applied for.

Dienone–Phenol Rearrangement of Sulphur-containing Derivatives of Steroids

James Y. Satoh,^{*,*} Amos M. Haruta,^{*} Toyoyuki Satoh,^{*} Keiichi Satoh,^{*} and Thomas T. Takahashi^b

^{*} Department of Chemistry, St. Paul's (Rikkyo) University, Nishi-Ikebukuro, Toshima-ku, Tokyo 171, Japan

^b Department of Chemistry, The Jikei University School of Medicine, Kokuryo, Chofu, Tokyo 182, Japan

Dienone–phenol rearrangement occurs in the reaction of an oxathiolane and dithiolane of 3-oxo-steroids with copper(II) bromide to give 4-methyl-19-norcholesta-1,3,5,10-trieno[1,2-*b*]-dihydroxathiine and -dihydrodithiine, respectively.

Reports on dienone–phenol type rearrangements in steroids have been limited almost entirely to highly unsaturated compounds, such as cross-conjugated dienone and trienone derivatives,¹ or labile compounds,^{2a} such as epoxy-derivatives.^{2b–d} No research of this type has been reported for sulphur-containing derivatives of steroids. We now report that rearrangement occurs in the reaction of oxathiolane derivatives of 3-oxo-steroids, which are stable saturated compounds, with copper(II) bromide to give 4-methyl-19-norcholesta-1,3,5,10-trieno[1,2-*b*]-dihydroxathiine, (2a).

The reaction was carried out as follows. A solution of the 3 α -O-oxathiolane (1a) (1 g) of 5 α -cholestan-3-one and cop-

per(II) bromide (2.5 g, 5 mol equiv.) in dioxane (40 ml) was refluxed for 3 h. The mixture was poured into ice-cold water and the resultant precipitate was filtered off. The filtrate was extracted with diethyl ether, and then the extract was chromatographed on silica-gel with light petroleum–benzene. Crystallization of the first fraction from methanol–ethanol gave 4-methyl-19-norcholesta-1,3,5,10-trieno[1,2-*b*]-dihydroxathiine (2a), 525 mg, 52%; m.p. 106–107.5°C; i.r. (KBr-disk, cm^{–1}) 863; ¹H n.m.r. (CDCl₃, δ) 2.15 (s, 3H, Ar-CH₃), 2.95–3.15 (br. t, 2H, S-CH₂), 4.15–4.45 (br. t, 2H, O-CH₂), 6.50 (s, 1H, Ar-H); *m/z* 440. In order to determine the configuration of the product of aromatization,

Evaluation of a Kinetic Method for Prostatic Acid Phosphatase with Use of Self-Indicating Substrate, 2,6-Dichloro-4-Nitrophenyl Phosphate

Andre A. Valcour, George N. Bowers, Jr., and Robert B. McComb¹

The purity, spectral characteristics, and rate of nonenzymatic hydrolysis of 2,6-dichloro-4-nitrophenyl phosphate (DCNPP) were determined. Rates of DCNPP hydrolysis by prostatic acid phosphatase (PAP) and erythrocytic acid phosphatase (EAP) (both EC 3.1.3.2) were measured in the absence and in the presence of various alcohols. 1,5-Pentanediol was the most effective transphosphorylation agent for specifically enhancing the activity of PAP. 1,4-Butanediol also enhanced PAP activity but markedly inhibited EAP activity. Bovine and human serum albumin preparations also accelerated the hydrolysis of DCNPP. DCNPP can be used for the continuous or multipoint-rate assay of PAP.

Additional Keyphrases: *alcohols and diols · albumin · enzyme activity · prostatic tissue and erythrocyte analysis*

2,6-Dichloro-4-nitrophenyl phosphate (DCNPP) has recently been introduced as a substrate for determination of acid phosphatase [orthophosphoric monoester phosphohydrolase (acid optimum); EC 3.1.3.2] in serum (1, 2).² The phenolic reaction product, 2,6-dichloro-4-nitrophenol (DCNP), formed during the assay at the pH optimum of acid phosphatases in human serum has a high molar absorptivity at 401 nm, a wavelength at which spectrophotometric measurements are both convenient and specific. Some substrates (2-4) that have been used to assay acid phosphatase are hydrolyzed to products that exhibit little absorption at pH 4-6 and thus require the addition of a second reagent before measurement. However, the hydrolysis of DCNPP can be monitored directly by a continuous or multipoint-rate method instead of requiring the indirect endpoint assays necessary with most other substrates. In addition, the DCNPP-based phosphatase assay exhibits simpler kinetics and yields a more stable product than does the kinetic method of Hillmann and its modifications (5-11) based on use of alpha-naphthyl phosphate-Fast Red TR.

Although nitrophenyl phosphatase assays are not specific for human prostatic acid phosphatase (PAP), this isoenzyme can be assayed in the presence of other acid phosphatases by taking advantage of the inhibitory effect of L-tartrate (10). After determinations of total acid phosphatase (TAP) and tartrate-insensitive acid phosphatase

(TIAP), PAP is calculated by difference ($PAP = TAP - TIAP$).

Here we report our studies on the analytical performance of assays of TAP and PAP with DCNPP as substrate. We assessed the correlation between PAP measurements made with 4-nitrophenyl phosphate (4NPP) and DCNPP as substrate, both with and without 1,5-pentanediol present as accelerator. As we report, the PAP measurements correlated well, but TAP determined with the two substrates did not correlate as well, owing to the heterogeneity of the patients' sera with regard to isoenzymes of acid phosphatase. The assay of TAP activity is further complicated by the fact that serum exhibits a residual DCNPP-phosphatase activity after heat treatment, which completely inactivates PAP and erythrocyte acid phosphatase (EAP). We show here that this tartrate-insensitive activity is associated with the albumin in serum, and that several albumin preparations quantitatively accelerate the rate of hydrolysis of DCNPP to DCNP and inorganic phosphate.

Materials and Methods

The substrate DCNPP was obtained from Toyobo Co. Ltd., Osaka, Japan (lots no. 8121A and 82220). DCNPP stock solutions were made up in 10 mmol/L acetic acid, final pH 3.2, and stored at 4 °C. The product DCNP was obtained from Toyobo Co. Ltd. (lot no. 8176) and from Aldrich Chemical Co., Milwaukee, WI 53233 (lot no. 43890-1). 4NPP was purchased from J. T. Baker Chemical Co., Phillipsburg, NJ 08865, and 4-nitrophenol (4NP), Standard Reference Material, was obtained from the National Institutes of Standards and Technology, Washington, DC 20234, as NIST/SRM 938. Human serum albumin (250 g/L) was purchased (lot no. 28015) from the American Red Cross, Washington, DC 20006. Bovine serum albumin was purchased from Sigma Chemical Co., St Louis, MO 63178 (lot no. 106F-0063).

Pretreatment of serum samples: Acid phosphatase in human serum was stabilized by acidification with 20 µL of 3.3 mol/L acetic acid per 2.0 mL of serum immediately after the serum was separated from the clot (10). To heat-denature the samples, we incubated them at 60 °C for 30 min.

Substrate characterization: Batches of DCNPP were dried for 12 h at 60 °C under reduced pressure and subjected to water content analysis with a Metrohm Karl Fischer-Automat E547 titrator (Brinkmann Instruments Inc., Westbury, NY 11590). Lithium, sodium, and potassium were measured with a Model 343 flame photometer (Instrumentation Laboratory, Inc., Lexington, MA 02173). Calcium and magnesium were measured on an AA-1475 Series atomic absorption spectrophotometer (Varian Associates, Palo Alto, CA 94303). Chloride was measured with a CMT10 Chloride Titrator (Radiometer, Copenhagen, Denmark). Total protein, albumin, and ammonia were measured by multilayer film analysis (Ektachem 700 Analyzer; Eastman Kodak Co., Rochester, NY 14650). The melting point of the dried materials was determined with a

Clinical Chemistry Laboratory, Department of Pathology, Hartford Hospital, Hartford, CT 06115.

¹ Address correspondence to this author.

² Nonstandard abbreviations: DCNP, 2,6-dichloro-4-nitrophenol; DCNPP, 2,6-dichloro-4-nitrophenyl phosphate; EAP, erythrocyte acid phosphatase; 4NP, 4-nitrophenol; 4NPP, 4-nitrophenyl phosphate; NMR, nuclear magnetic resonance; PAP, prostatic acid phosphatase; ppm, parts per million; TAP, total acid phosphatase; TCA, trichloroacetic acid; TIAP, tartrate-insensitive acid phosphatase; NIST, National Institute of Standards and Technology; and SRM, Standard Reference Material.

Received January 20, 1989; accepted March 17, 1989.

Thomas Hoover capillary boiling-point apparatus (Thomas Scientific, Swedesboro, NJ 08085).

For thin-layer chromatography we used glass plates precoated with Silica Gel G containing ammonium sulfate, 50 g/kg (Analtech, Newark, DE 19711). Chromatograms were developed with a mixture of *n*-butanol, acetic acid, and water (60/15/25 by vol), and sample components were made visible by charring on a hot plate. ^1H NMR (nuclear magnetic resonance) spectra were obtained at 500 MHz with a General Electric NMR spectrometer operating in the Fourier transform mode, at the University of Santa Barbara, Santa Barbara, CA 93107 (courtesy of Lauren Brown of J.B.L. Scientific, San Luis Obispo, CA 93401).

The molar absorptivity of DCNP in 60 mmol/L sodium citrate, pH 5.5, was determined with a Cary Model 219 dual-beam spectrophotometer (Varian Associates). We verified its wavelength accuracy with a holmium oxide wavelength standard (12) and checked the photometric accuracy with NIST/SRM 938 (13). Absorption was measured against air in a single quartz cell, NIST/SRM 932, whose pathlength was certified to 5 parts per 100 000 (14). The absorption values of various lots of DCNP were determined and subtracted from the absorption of solvent in the same cuvette.

The purity of the various lots of DCNPP was estimated by measuring the number of moles of DCNP and inorganic phosphate produced by the complete nonenzymatic hydrolysis of a carefully measured dry mass of DCNPP dissolved in 10 mmol/L NaOH. To calculate the expected yield, we used relative molecular masses of 208.0 for DCNP and 288.3 for DCNPP (free phosphoric acid). DCNP concentration was measured by absorbance at 401 nm after 50-fold dilution in 60 mmol/L citrate, pH 5.5, according to the molar absorptivity value determined for DCNP.

The inorganic phosphate produced from the hydrolysis of DCNPP was quantified at 37 °C in a Cobas-Bio centrifugal analyzer (Roche Diagnostics, Nutley, NJ 07110) with an inorganic phosphorus kit (Roche Diagnostics, cat. no. 44266). The absorbance of the unreduced phosphomolybdate complex produced in this assay is measured at 340 nm and is subject to interference by DCNP. To avoid this spectral interference, we modified the assay to include a reduction step before measurement of the phosphomolybdate blue complex at 620 nm (15). First, protein from the samples was removed by precipitation in 1.25 mol/L TCA, then 10 μL of supernate, 10 μL of diluent water, and 200 μL of Roche "Inorganic Phosphate Reagent" were mixed by the Cobas-Bio. After a 1-min incubation, 40 μL of a mixture containing, per liter, 1.46 mol of sodium bisulfite, 40 mmol of sodium sulfite, and 20 mmol of 1-amino-2-naphthol-4-sulfonic acid was added, followed by 20 μL of diluent water. Absorbance at 620 nm was measured after a 12-min incubation. DCNPP, DCNP, 1,5-pentanediol, and TCA did not interfere with assay of inorganic phosphorus by this method.

We used "high-performance" liquid chromatography, with detection at 205 nm, to detect impurities in both DCNPP and DCNP. A mixture of 50 parts of CH_3CN and 50 parts of 10 mmol/L H_3PO_4 (pH 2.0) was pumped through an HS-5 C_{18} column (Perkin-Elmer Corp., Oak Brook, IL 60521) at 1.0 mL/min. Two lots of DCNPP were analyzed directly and after complete nonenzymatic hydrolysis in 10 mmol/L NaOH. Two lots of DCNP were analyzed in an analogous manner.

Nonenzymatic hydrolysis: We determined the rate of

hydrolysis of DCNPP at 30 °C as a function of pH. We prepared a set of sodium citrate buffers (60 mmol/L), with pH values ranging from 3.0 to 9.0. Fifty microliters of a DCNPP stock solution (6 mmol/L) and 10 μL of diluent water were mixed with 250 μL of sodium citrate buffer by the Cobas-Bio. The increase in absorbance at 401 nm was measured after a 10-min incubation. The molar absorptivity of DCNP in the buffers was also determined with the Cobas-Bio. Fifty microliters of 0.25 mmol/L DCNP and 10 μL of diluent water were mixed with 250 μL of sodium citrate buffer. The rate of nonenzymatic hydrolysis of DCNPP at a given pH was calculated, using the molar absorptivity of DCNP determined at the same pH.

Acid phosphatase assays: The enzymatic hydrolysis of DCNPP at 30 °C was measured with a procedure modified from Teshima et al. (1). We measured TAP activity in 60 mmol/L citrate, pH 5.5, and TIAP activity in 60 mmol/L citrate, 26.7 mmol/L tartrate, pH 5.5 (final reaction-mixture concentrations). The production of DCNP was monitored at 401 nm with the Cobas-Bio. The procedure involved an initial 3-min incubation of a mixture of 20 μL of serum, 10 μL of diluent water, and 200 μL of buffer. The reaction was started by adding 50 μL of 6 mmol/L DCNPP and 20 μL of diluent water, and the absorption was measured at 60-s intervals for 10 min. The final volume fraction of serum was 0.067 and the substrate concentration in the final reaction mixture was 1 mmol/L.³

We determined the effect of a variety of alcohols and diols on the hydrolysis of DCNPP by acid phosphatase isoenzymes. Water-soluble alcohols were mixed with the citrate and citrate/tartrate buffers at concentrations ranging from 200 to 1000 mmol/L (final reaction-mixture concentrations, 134 to 667 mmol/L). Alcohols that were not as water soluble as 200 mmol/L were tested at the limit of their solubility.

The enzymatic hydrolysis of 4NPP was measured with the Cobas-Bio by a modification of the method of Jacobsson (10). Twenty microliters of serum and 20 μL of diluent water were mixed with 250 μL of buffer containing substrate. The final reaction concentrations of buffer and substrate were identical to those used in the DCNPP assay. After a fixed period of incubation at 30 °C, the reaction mixture was made alkaline with 75 μL of 2 mol/L NaOH and the absorption at 401 nm was determined. A blank was measured to correct for absorbing compounds in the sample by adding the NaOH before adding the sample.

Tissue extracts: We prepared an extract of prostatic tissue, as previously described (16), and a hemoglobin-free erythrocyte extract from outdated whole blood (17). Plasma and leukocytes were decanted from heparinized blood after centrifugation (10 000 $\times g$, 4 °C, 10 min), and the precipitated erythrocytes were lysed at 4 °C in a 2.5-g/L solution of Brij-35 surfactant (Pierce Chemical, Rockford, IL 61105) in de-ionized water. The lysate was centrifuged (15 000 $\times g$, 4 °C, 10 min), and the supernate was dialyzed against several volumes of potassium phosphate buffer (5 mmol/L, pH 6.0). The dialysate was applied to a column of diethylaminoethyl Sephadex (Pharmacia Fine Chemicals, Piscataway, NJ 08854) and washed with the potassium phosphate buffer until all of the color was eluted from the column. The acid phosphatase activity was then eluted from the column with 600 mL of 5 mmol/L potassium

³ Based on a formula weight of 288.3, a value that appears to be 30% low (see Discussion).

phosphate, 300 mmol/L NaCl, pH 7.0; insignificant amounts of acid phosphatase activity were removed with a further 600 mmol/L NaCl wash. The extract was concentrated with an ultrafiltration apparatus equipped with a PM-10 membrane (Amicon Division, WR Grace & Co., Danvers, MA 01923).

Results

Characterization of Substrate and Product

Both lots of DCNPP eluted from the HS-5 C₁₈ column as a symmetrical major peak followed by a second, smaller peak (Figure 1). The smaller peak had a retention time identical to that of DCNP. The completely hydrolyzed substrate was eluted as a single peak with a retention time identical to DCNP. There was no residual material with the retention time of the major DCNPP peak. Meticulous spectrophotometry at 401 nm and the assay of inorganic phosphate indicated that equal molar amounts of DCNP and phosphate were produced by the complete hydrolysis of DCNPP in 10 mmol/L NaOH. The yield of these products was 73.3% and 72.4%, respectively, of that calculated assuming that DCNPP was in the free-acid form.

Because the pH of a 6 mmol/L DCNPP solution in de-ionized water is 2.73, it was reasonable to suspect that this material is in the free-acid form. We found negligible amounts of the common cations in either substrate or the reference standard DCNP preparations (Table 1). Contamination of these materials with water and free chloride was negligible. The fairly sharp melting points listed in Table 1 suggest that both product and substrate are reasonably pure. However, preliminary analysis of the substrate by

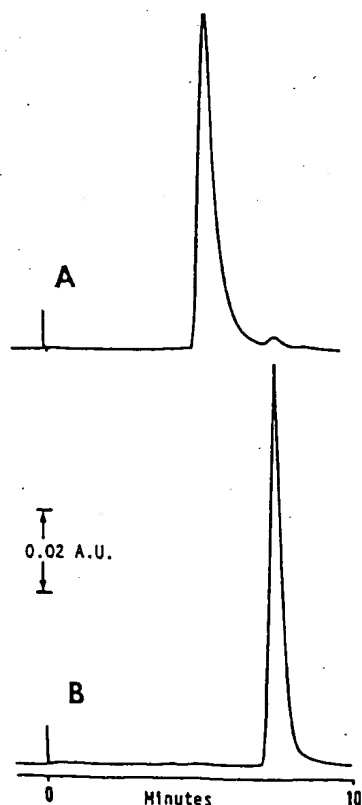


Fig. 1. HPLC of DCNPP and DCNP with detection at 205 nm. A, DCNPP as received (Toyobo, lot no. 8176); B, hydrolyzed DCNPP (Toyobo, lot no. 8121A). A.U., absorbance unit

Table 1. Materials Analysis

Composition, mg/g	DCNPP	DCNP
Water	<5	<5
Sodium	<2	<0.1
Potassium	<0.01	<0.01
Lithium	<2	<0.01
Magnesium	<2	<2
Calcium	<2	<2
Ammonia	<0.2	<0.05
Free chloride	<2	<1
Other characteristics		
Melting point, °C	152–154	121–123
λ_{\max} , nm	292	401
ϵ , L · mol ⁻¹ · cm ⁻¹	45 ^a (6100) ^b	15 370 ± 80 ^c
Purity by HPLC, %	98	100

^a $\epsilon_{401 \text{ nm}}$ for DCNPP; all lots were contaminated with small amounts of DCNP (see Fig. 2).

^b $\epsilon_{292 \text{ nm}}$ for DCNPP based on a relative molecular mass of 288.8 and corrected for above impurities. Other evidence (see Results) indicates that this material is not the pure free acid, in which case the $\epsilon_{292 \text{ nm}}$ will be approximately 30% higher than reported here.

^c $\epsilon_{401 \text{ nm}} \pm 1 \text{ SD}$ for DCNP lots from Aldrich and Toyobo.

^d Based on relative peak heights in Fig. 1 and estimates of DCNP concentrations from external standards.

thin-layer chromatography on silica gel indicated that it contained at least one contaminating organic component, which may account for the discrepancy in product yield.

The ¹H NMR spectrum of DCNP in *d*₆-dimethyl sulfoxide (not shown) indicates that it is free of protonated organic contaminants. The spectrum has a single low-field resonance at 8.25 ppm, corresponding to the two symmetric protons on the aromatic ring of DCNP. On the other hand, the spectrum of DCNPP has a sharp low-field resonance at 8.25 ppm and a broader resonance at 7.96 ppm. The spectrum also contains a complex set of resonances at high field (1 to 3 ppm), which correspond to approximately 18 aliphatic protons and have a total integrated area that is double that of the aromatic protons. The aliphatic component(s) were not identified but probably correspond to an organic counter-ion to the DCNPP.

Figure 2 depicts the ultraviolet-visible spectra of DCNP and DCNPP in 60 mmol/L sodium citrate, pH 5.5. The molar absorptivities under these conditions are listed in Table 1. There were no significant spectral differences between the lots of DCNPP or the lots of DCNP. The spectrum of DCNP under these conditions is similar to that at pH 5.0 in acetate buffer (1). DCNPP has an absorbance maximum at 292 nm and little absorbance at 401 nm.

Figure 3 shows the rate constant for the nonenzymatic hydrolysis of DCNPP at 30 °C as a function of pH. The hydrolysis of DCNPP is maximal when its phosphate group is fully ionized (pH > 7) and the rate of hydrolysis at pH 5.5 is 10-fold that at pH 3.5. DCNPP stock solutions held at pH 3.2 and 4 °C underwent 0.05% nonenzymatic hydrolysis per day. The powdered substrate stored undessicated at room temperature was slowly hydrolyzed to product (approximately 0.4% per year).

Method Development

PAP hydrolyzed DCNPP 1.28 times as rapidly as 4NPP, and EAP hydrolyzed DCNPP 0.58 times as rapidly. Tartrate at a concentration of 60 mmol/L inhibited the DCNPP-phosphatase activity in the prostate extract by

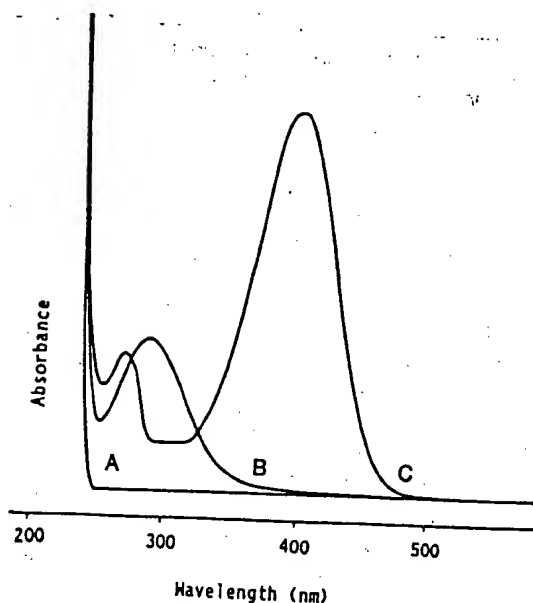


Fig. 2. Spectra of substrate and product
A, buffer alone: 60 mmol/L citrate, pH 5.5; B, buffer plus DCNPP, 35 μ mol/L;
C, buffer plus DCNP, 35 μ mol/L

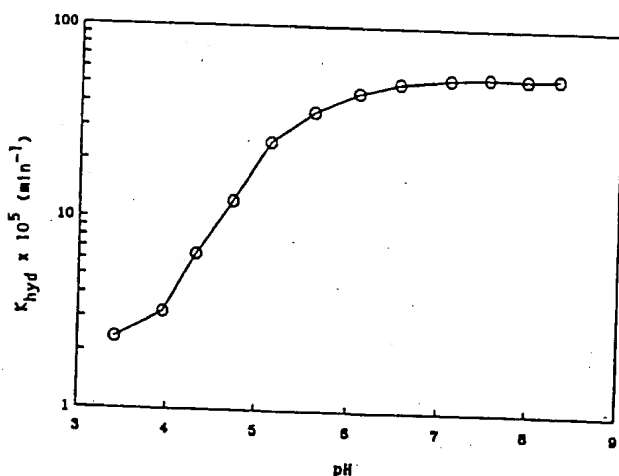


Fig. 3. pH-rate-constant profile for nonenzymatic hydrolysis of DCNPP
 K_{hyd} = first-order rate constant at 30 °C

>98%, in agreement with previous work (1), but did not affect DCNPP hydrolysis by the erythrocyte extract. Citrate and acetate support PAP activity to a greater extent than several other buffers (Figure 4). We chose to use citrate buffer because it is widely used in other procedures for acid phosphatase, particularly the procedure of Jacobson (10), in which 4NPP is the substrate. The pH optimum for the PAP-catalyzed hydrolysis of DCNPP in citrate buffer is between 5.0 and 5.5, similar to the pH optimum in acetate buffer (1). At pH 5.5, the rate of nonenzymatic hydrolysis of DCNPP is about four times the rate of enzymatic hydrolysis at the upper reference limit of PAP (2.0 U/L). Prostatic enzyme activity is unaffected by increasing NaCl and KCl concentration, but erythrocyte enzyme activity is markedly inhibited (data not shown).

We determined the degree to which a number of alcohols accelerated the production of DCNP (Table 2). 1,5-Pentenediol activated PAP to the greatest extent. The ratio of DCNP to inorganic phosphate produced in the absence of alcohols was 1.00, but the addition of 1,5-pentenediol

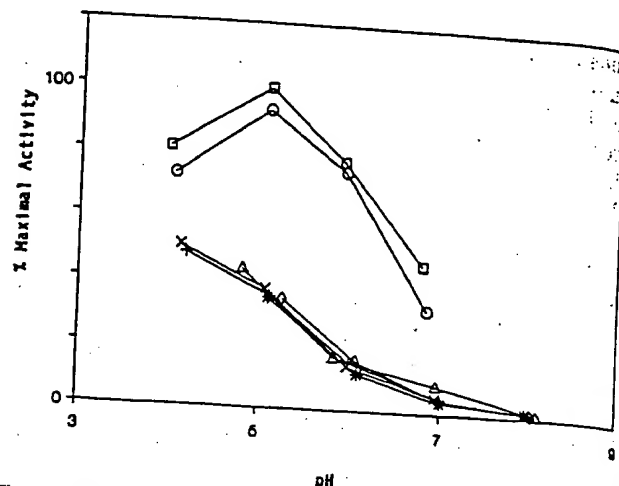


Fig. 4. Effect of buffers (1 mol/L) on PAP-catalyzed hydrolysis of DCNPP

DCNPP-based phosphatase activity as a function of pH in: acetate (\square), citrate (\circ), *N,N*-bis[2-hydroxyethyl]glycine (Δ), bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane (+), *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (\times), hydroxylamine (\times), and *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid (\star). Previous work (1) and our confirmatory experiments (not shown) indicate that the pH optimum for DCNPP hydrolysis of prostate extract lies between pH 5.0 and 5.5

increased this ratio to 2.00. While the production of DCNP increased by 1.94-fold, the yield of phosphate decreased by 0.81-fold. These data indicate that 1,5-pentenediol accelerates the production of DCNP by action as a substrate for transphosphorylation. This mechanism has been previously described in acid phosphatase (18) and alkaline phosphatase, where the best accelerators are amino alcohols (19).

DCNPP-Phosphatases in Human Serum

We performed precision studies with Hartford Hospital's human serum control material (16), which is supplemented with prostate extract. The TAP assay had a within-run ($n = 12$) CV of 1.78% (mean = 23.7 U/L) and a between-day CV ($n = 12$) of 3.64% (mean = 23.6 U/L). The PAP assay had a within-run ($n = 12$) CV of 1.97% (mean = 15.9 U/L) and a between-day ($n = 12$) CV of 3.71% (mean = 15.8 U/L). DCNPP- and 4NPP-based assays of TAP and PAP in patients' serum samples correlate well, both with and without 1,5-pentenediol in the DCNPP-based assay (Figure 5), with the DCNPP-based TAP assay showing a consistent positive bias. In our investigation of the source of this bias, we found that serum samples that are incubated at 60 °C for >4 h have DCNPP-phosphatase activity. No 4NPP-phosphatase activity remains in these samples. Furthermore, the DCNPP- and 4NPP-phosphatase activities of both the prostatic and erythrocyte extracts are completely inactivated in less than 10 min at 60 °C. The heat-stable, DCNPP-phosphatase activity of serum correlates weakly with albumin concentration (Figure 6; $r = 0.6816$) and less so with total protein concentration ($r = 0.4331$). Commercial preparations of bovine and human serum albumin increase the rate of DCNPP hydrolysis both before and after incubation at 60 °C. This albumin-associated activity is proportional to albumin concentration at the relatively low final reaction concentrations that are produced when patients' samples are assayed. At higher reaction mixture concentrations the DCNPP-phosphatase activity of albumin levels off at approximately 25 U/L (Figure 7). Equimolar concentrations of DCNP and phosphate are produced,

Table 2. Activation of Acid Phosphatase by Alcohols

Alcohol	Prostate extract		Erythrocyte extract	
	Alcohol concn, ^a mmol/L	Relative activity, ^b %	Alcohol concn, ^a mmol/L	Relative activity, ^b %
None	—	100.0	—	100.0
Methanol	667 ^d	123.1	667 ^d	143.1
Ethanol	267	120.7	667 ^d	146.7
Ethylene glycol	667 ^d	121.2	667 ^d	145.1
1-Propanol	400	139.3	667 ^d	186.9
2-Propanol	267	104.5	267	105.1
1,2-Propanediol	667 ^d	147.8	667 ^d	161.3
1,3-Propanediol	400	120.3	667 ^d	121.9
Glycerol	267	100.9	667 ^d	133.7
1-Butanol	400	170.2	400	151.2
1,3-Butanediol	400	147.8	667 ^d	135.6
1,4-Butanediol	667 ^d	175.7	267 ^e	5.5
1-Pentanol	— ^c	163.9	— ^c	160.8
1,5-Pentanediol	267	198.7	133	123.3
1,4-Pentanediol	267	120.6	267 ^e	65.6
1,2-Pentanediol	267	156.4	667 ^d	288.6
2R,4R-Pentanediol	267	104.9	267	105.1
Cyclopentanol	267 ^e	90.9	267 ^e	42.7
1-Hexanol	— ^c	133.7	— ^c	119.4
1,2-Hexanediol	133	168.0	267	174.2
1,5-Hexanediol	400	151.8	667 ^d	145.1
1,6-Hexanediol	267	163.9	267	160.4
2,5-Hexanediol	133	108.5	267 ^e	85.0
1,4-Cyclohexanediol	400	123.7	267 ^e	92.4
3-Methyl-1-butanol	— ^c	155.0	— ^c	97.7
cis-1,2-Cyclohexane dimethanol	133	181.0	267 ^e	91.6
1,5-Pentanediol and 1,4-butanediol	267 ^f	214.0	267 ^f	24.4

^a Final reaction concentration of alcohol that produced the maximum acceleration of DCNPP-based phosphatase activity.

^b Activity in presence of maximally effective concentration of alcohol relative to activity in absence of alcohol.

^c Assay performed at limit of solubility of these alcohols (<133 mmol/L).

^d Highest concentration of alcohol tested in cases where activity continued to increase with increasing concentration.

^e Alcohol exhibiting inhibitory effect where activity continues to decrease with increasing concentration.

^f Both buffers at a final reaction concentration of 267 mmol/L.

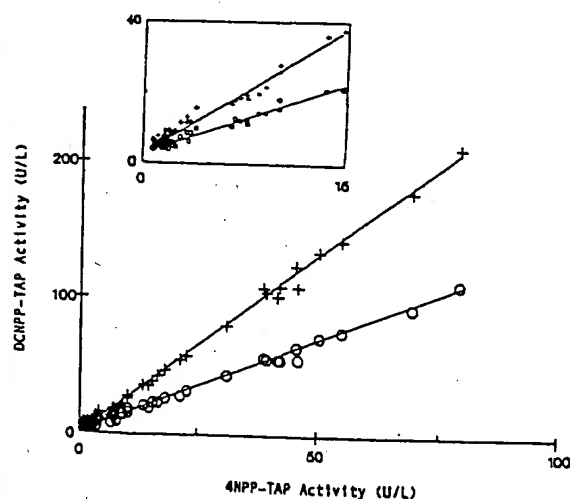


Fig. 5. Correlation of TAP activity with two substrates

TAP correlation without 1,5-pentanediol in either assay (O): $y = 1.263x + 4.443$ U/L ($n = 67$, $r = 0.9944$); with 1,5-pentanediol (267 mmol/L final concentration in DCNPP assay (+): $y = 2.502x + 3.225$ U/L ($n = 67$, $r = 0.9960$

The correlation for PAP (not shown) was similar except for the absence of a significant positive bias in the intercept. PAP correlation without 1,5-pentanediol in either assay: $y = 1.298x + 0.342$ U/L ($n = 67$, $r = 0.9961$); with 1,5-pentanediol (267 mmol/L) in DCNPP assay: $y = 2.562x + 0.094$ U/L ($n = 67$, $r = 0.9970$)

indicating that albumin is acting as a catalyst and not as a transphosphorylation acceptor. The rate of albumin-catalyzed DCNPP hydrolysis is independent of pH up to at least

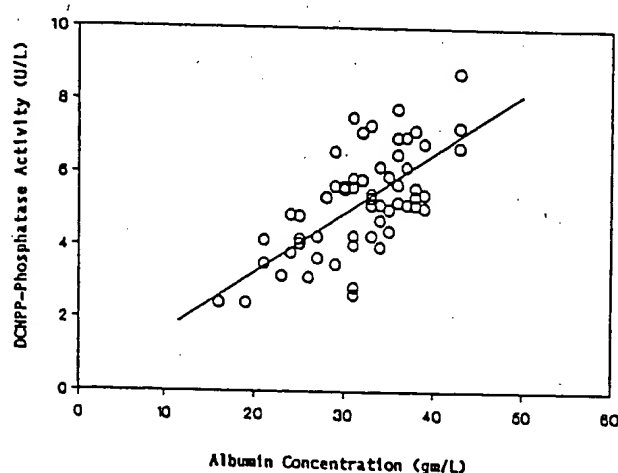


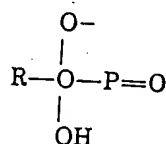
Fig. 6. DCNPP-based phosphatase activity of heat-denatured serum. The residual DCNPP-based phosphatase activity of serum samples incubated at 60 °C for 30 min vs serum albumin concentration of the heat-treated sample: $y = 1.636x + 0.003$ U/L ($n = 59$, $r = 0.6816$)

pH 8.0 and is not affected by any of the alcohols tested. Albumin has no detectable 4NPP-phosphatase activity.

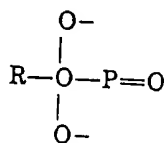
Discussion

Many laboratory instruments are designed to measure analytes in a multipoint-rate mode, so the use of DCNPP offers a distinct technical advantage over many other substrates currently used for acid phosphatase (2-4). The hydrolysis product DCNP is nearly fully ionized at the pH optimum of acid phosphatase (1), yielding high absorbance at a convenient wavelength. The absence of lag phase and the stability of the colored product are unique qualities of the new assay that are not shared with the kinetic method of Hillmann (6) and its modifications. While the substrate can be obtained in what appears to be a consistent quality, as judged by the analysis of two lots from Toyobo, it is not a simple, pure material. Hydrolytic conversion studies indicate that the exact atomic formula of the material needs to be established. Although complete hydrolysis of DCNPP yields equal amounts of DCNP and phosphate, the product yield is about 28% less than that calculated, assuming the DCNPP is an anhydrous free acid. The thin-layer chromatographic and proton magnetic resonance data suggest that this discrepancy can be accounted for by one or more unidentified organic components. However, we found no evidence that the presence of these unidentified materials adversely affects the utility of the substrate for the assay.

Extra care must be taken in both the preparation and storage of DCNPP solutions, because its phosphate ester is unstable at pHs >3.5. The dry substrate should be desiccated and stored at 4 °C, and substrate stock solutions should be made up in 10 mmol/L acetic acid to ensure a low pH. Kirby and Varvoglis (20) found that the rate of non-enzymatic hydrolysis of the phosphate esters of many phenols increases with decreasing pK_a' of the phenol. They identified two distinct types of pH-rate profile for nonenzymatic hydrolysis of phosphate esters. If the pK_a' of the leaving-group alcohol exceeds 5.5 (as is the case for, e.g., 4NPP), the rate of hydrolysis is maximal when the phosphate is in the monoanion



form (pH 4.0). Alternatively, the phosphate esters of phenols with $pK_a' < 5.5$ (e.g., DCNPP) exhibit maximal hydrolysis when the phosphate is in the dianion



form (pH >5.0). Thus, aqueous solutions of DCNPP must be stored at a low pH and added to the reaction mixture at the start of the assay. This presents little problem in a two-reagent assay system as currently supplied by Toyobo (1), but it may complicate the use of DCNPP in single-reagent systems.

The ability of alcohols, particularly ethanol, to accelerate

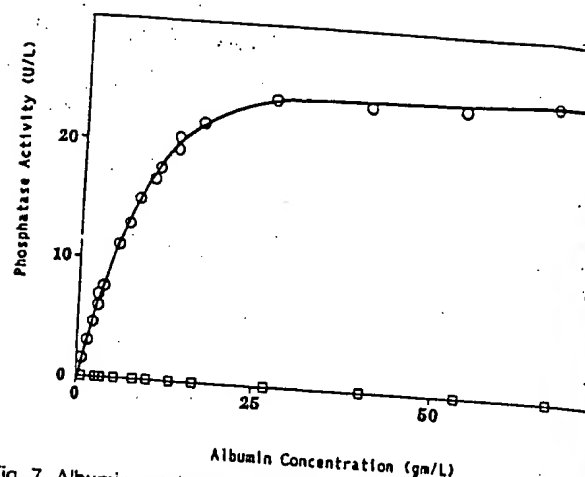


Fig. 7. Albumin-catalyzed hydrolysis of DCNPP and 4NPP. The 4NPP- (□) and DCNPP-based (○) phosphatase activity of human serum albumin plotted as a function of albumin concentration in the final reaction mixture. The range of sample albumin concentrations plotted on the abscissa of Fig. 6 (0-60 g/L) corresponds to the range of final reaction concentrations of 0-4 g/L in the above figure.

the activity of PAP was first noted by Appleyard in 1921. 1-Pentanol was later shown to markedly increase the 4NPP-phosphatase activity of prostate extracts (11, 22). Poindexter et al. (8) showed that the more soluble 1,4-pentanediol was an even more effective accelerator of PAP activity. We have undertaken a thorough analysis of the effects of a series of alcohols on the hydrolysis of DCNPP by PAP and EAP. All aliphatic alcohols with one or more terminal hydroxy groups accelerate the PAP activity to some extent. 1,5-Pentanediol at a final reaction concentration of 267 mmol/L is the most effective accelerator of PAP activity and nearly doubles the sensitivity of the assay. Most of the aliphatic terminal alcohols also increase the rate of hydrolysis of DCNPP by EAP. However, the two 1,4-diols we tested inhibited the EAP activity. It should be noted that an equimolar mixture of 1,5-pentanediol and 1,4-butanediol in citrate buffer enhances PAP activity to the same or greater extent than 1,5-pentanediol alone, but markedly inhibits EAP activity. If 1,4-butanediol has an inhibitory effect on other acid phosphatase isoenzymes and the albumin-associated activity can be inhibited, a specific assay to measure PAP in a single reaction might be developed.

The heat-stable DCNPP-phosphatase activity in patients' samples, which seems to have been overlooked in a previous report (1), will limit the utility of this new substrate for the measurement of TAP. This activity varies with serum albumin concentration, and it adds a variable positive bias to the TAP values. To the best of our knowledge, similar albumin-associated phosphatase activity has not been reported for any of the other substrates used for the assay of acid or alkaline phosphatase. However, albumin reportedly is associated with the enzymatic hydrolysis of aromatic esters of aliphatic acids (23-26). This arylesterase activity is resistant to physostigmine, a cholinesterase inhibitor, and has been demonstrated in albumin preparations produced by various methods (25, 26). The activity is also heat resistant (26) but may differ from the DCNPP-phosphatase activity in that it exhibits a pH optimum between 7.9 and 10 (25, 26).

The positive bias produced by albumin is relatively large as compared with the normal reference interval for TAP (27), and we found no simple means of blank correction.

Blank correction by heat denaturation at 60 °C is impractical, and acidified serum samples tend to gel at this temperature.

Despite the shortcomings of the TAP assay, DCNPP is an excellent substrate for the measurement of PAP as the tartrate-sensitive component of DCNPP-phosphatase activity ($PAP = TAP - TIAP$). This procedure provides the same insensitivity to nonprostatic acid phosphatase isoenzymes, including the EAP isoenzymes and albumin, as the 4NPP-based assay does. The inclusion of the transphosphorylation agent, 1,5-pentanediol, in the DCNPP assay specifically activates the PAP relative to EAP and doubles the sensitivity of the PAP assay. The simplicity, high sensitivity, and precision of this new assay suggest that it may well become the PAP assay of choice in many laboratories. We believe that it may be possible to develop a candidate Reference Method for PAP, with DCNPP as substrate. A Reference Method might include 1,5-pentanediol to increase sensitivity and 1,4-butanediol to inhibit EAP. In addition, the substrate used in a Reference Method should be fully characterized and readily available from more than one source. Fortunately, an alternative supplier (JBL Scientific, San Luis Obispo, CA 93401) has synthesized DCNPP and is currently testing various lots. However, extensive studies of the DCNPP-phosphatase activity of albumin and the other isoenzymes of acid phosphatase found in patients' sera will be required before a candidate Reference Method can be rationally proposed.

References

1. Teshima S, Hayashi Y, Ando M. Determination of acid phosphatase in biological fluids using a new substrate 2,6-dichloro-4-nitrophenyl phosphate. *Clin Chim Acta* 1987;168:231-8.
2. Roy AV, Brower ME, Hayden JE. Sodium thymolphthalein monophosphate: a new acid phosphatase substrate with greater specificity for the prostatic enzyme in serum. *Clin Chem* 1971;17:1093-102.
3. Babson AL, Read PA, Phillips GE. The importance of the substrate in assays of acid phosphatase in serum. *Am J Clin Pathol* 1959;32:83-8.
4. Fishman WH, Lerner F. A method for estimating serum acid phosphatase of prostatic origin. *J Biol Chem* 1952;200:89-97.
5. Gundlach G, Muhlhausen B. Untersuchungen zur Kupplung des 1-Naphthols mit Fast-Red-TR. *J Clin Chem Clin Biochem* 1980;18:603-10.
6. Hillmann G. Fortlaufend photometrische Messung der Säuren Phosphatase-Aktivität. *Z Klin Chem Klin Biochem* 1971;3:273-4.
7. Escribano J, García-Carmona F, García-Canovás F, Iborra JL, Lozano JA. Kinetic analysis of chemical reactions coupled to an enzymic step; applications to acid phosphatase assay with Fast Red. *Biochem J* 1984;223:633-8.

8. Poindexter C, Ervin K, Rice EG. A kinetic acid phosphatase (Hillmann) procedure of improved sensitivity [Abstract]. *Clin Chem* 1980;26:1009.
9. Renze B, Edwards JB. An optimized continuous-monitoring procedure for semiautomated determination of serum acid phosphatase activity. *Clin Chem* 1976;22:2025-8.
10. Jacobsson K. The determination of tartrate-inhibited phosphatase in serum. *Scand J Clin Lab Invest* 1960;12:367-80.
11. Gallati H, Roth M. Aktivierung der säuren Prostataphosphatase durch 1-Pentanol. *J Clin Chem Clin Biochem* 1976;14:581-7.
12. McNiery J, Slavin W. A wavelength standard for ultraviolet-visible-near-infrared spectrophotometry. *Appl Opt* 1962;1:365-72.
13. Bowers Jr GN, McComb RB, Christensen RG, Schaffer R. High-purity 4-nitrophenol: purification, characterization, and specifications for use as a spectrophotometric reference material. *Clin Chem* 1980;26:724-9.
14. Mavrodineanu R, Lazar JW. Standard Reference Materials: standard quartz cuvettes for high-accuracy spectrophotometry. *Clin Chem* 1973;19:1053-7.
15. Crouch SR, Malmstadt HV. A mechanistic investigation of molybdenum blue method for determination of phosphate. *Anal Chem* 1967;39:1084-9.
16. Bowers Jr GN, Burnett RW, McComb RB. Preparation and use of human serum control materials for monitoring precision in clinical chemistry. *Clin Chem* 1975;21:1830-6.
17. Fenton MR, Richardson KE. Isolation of three acid phosphatase isozymes from human erythrocytes. *Arch Biochem Biophys* 1967;120:332-7.
18. Ostrowski W, Barnard EA. Evidence for a phosphoryl-enzyme intermediate in the catalytic reaction of prostatic acid phosphatase. *Biochemistry* 1973;12:3893-8.
19. McComb RB, Bowers Jr GN. Study of optimum buffer conditions for measuring alkaline phosphatase activity in human serum. *Clin Chem* 1972;18:97-104.
20. Kirby AJ, Varvoglis AG. The reactivity of phosphate esters. Monoester hydrolysis. *J Am Chem Soc* 1967;89:415-23.
21. Appleyard J. The effect of alcohols on the hydrolysis of sodium phenolphthalein diphosphate by prostatic extracts. *Biochem J* 1948;42:596-8.
22. Sawada H, Asano S, Maruyama H, Shinoda M, Aoki Y. Studies on human prostatic acid phosphatase. III. The effects of alcohols on human erythrocyte acid phosphatase and on human prostatic acid phosphatase. *Chem Pharm Bull* 1980;28:3466-72.
23. Casida JE, Augustinsson K-B. Reaction of plasma albumin with 1-naphthyl-N-methylcarbamate and certain other esters. *Biochim Biophys Acta* 1959;36:411-26.
24. Tove SB. The esterolytic activity of serum albumin. *Biochim Biophys Acta* 1962;57:230-5.
25. Wilde CE, Kekwick RGO. The arylesterases of human serum. *Biochem J* 1964;91:297-307.
26. Wolfbeis OS, Gurakar A. The effect of fatty acid chain length on the rate of arylester hydrolysis by various albumins. *Clin Chim Acta* 1987;164:329-37.
27. Schiele F, Artur Y, Floc'h AY, Siest G. Total, tartrate-resistant, and tartrate-inhibited acid phosphatases in serum: biological variations and reference limits. *Clin Chem* 1988;34:685-90.

DAIRY PRODUCTS

Fluorometric Determination of Alkaline Phosphatase in Fluid Dairy Products: Collaborative Study

RICHARD M. ROCCO

Advanced Instruments, Inc., 1000 Highland Ave, Needham Heights, MA 02194

Collaborators: J. Beyer; R. L. Bradley, Jr; B. Burwell; T. Cronau; R. Cyr; D. Elliott; L. Hensel; J. Jaworski; L. Justis; D. Kleyn; L. Lengyel; M. Lundberg; C. Mayfield; J. Scott; J. Siebodnik; C. Strayer; R. K. Stuckey; B. Thornhill; T. Way; H. M. Wehr

Official methods for the measurement of alkaline phosphatase (ALP) in dairy products use either phenyl phosphate or phenolphthalein monophosphate as substrate. Quantitation of results requires butanol extraction of the indophenol (Scharer) or 3-h dialysis of the liberated phenolphthalein (Rutgers). The Advanced Fluorophos® assay is based on a self-indicating substrate which, when acted upon by ALP, loses a phosphate radical and becomes a highly fluorescent compound. The rate of fluorophore formation is monitored for 3 min in a fluorometer and the enzyme activity in mU/L is calculated. Eight laboratories participated in a collaborative study to evaluate the Fluorophos assay for determining ALP activity in whole milk, sklm milk, chocolate milk, and cream (half and half). The comparative method was the AOAC quantitative phenyl phosphate method, 16.121-16.122 (14th Ed.). Mixed herd raw milk was added to pasteurized samples at 0.05, 0.1, and 0.2% (v/v). Method performance at 0.1% (v/v) added raw milk as measured by repeatability and reproducibility standard deviations (s_r and s_R) and relative standard deviations (RSD_r and RSD_R), respectively, were: whole milk, $s_r = 21.7\%$, $s_R = 34.6\%$, RSD_r = 4.4%, RSD_R = 7.0%; sklm milk, $s_r = 19.2\%$, $s_R = 31.4\%$, RSD_r = 3.8%, RSD_R = 6.2%; chocolate milk, $s_r = 27.6\%$, $s_R = 45.8\%$, RSD_r = 5.3%, RSD_R = 8.8%. The method has been adopted official first action by AOAC for determination of alkaline phosphatase in whole milk, sklm milk, and chocolate milk.

The measurement of alkaline phosphatase (ALP, EC 3.1.3.1) has been used to assess the completeness of pasteurization in dairy products for over 50 years (1). Current AOAC methods for ALP use either phenyl phosphate (16.121-16.122, 14th Ed.; 979.13, 15th Ed.) or phenolphthalein monophosphate (16.116-16.120, 14th Ed.; 972.17, 15th Ed.) as the colorimetric substrate (2). Quantitative results with these substrates require isolation of the enzymatically formed product from interfering turbidity prior to spectrophotometric readings. Modifications of the phenyl phosphate method use either butanol extraction of the blue indophenol (16.121) or precipitation of proteins and lipids with zinc and barium salts (16.112-16.114, 14th Ed.; 946.01, 15th Ed.). Quantitation in the phenolphthalein monophosphate method

requires a 3-h dialysis of the liberated red phenolphthalein into water (16.116, 14th Ed.; 972.17, 15th Ed.). Despite their complexity and long incubation times, current ALP methods have served the dairy industry well and have aided in monitoring milk quality throughout the world for many years.

The purpose of the present study was to collaboratively examine a new fluorometric assay for ALP in dairy products (3). The method is based on a fluorometric substrate called Fluorophos®, which, when acted upon by ALP, is converted to a highly fluorescent product. This fluorometric quantitative assay is the first dairy product ALP test that permits continuous and direct measurement of the released reaction product from a self-indicating substrate. The assay requires only 1 working reagent and is complete in 3 min. Use of this fluorometric substrate eliminates the interferences and non-specificity encountered in colorimetric assays and avoids the need for dialysis, butanol extraction, or protein precipitation. Fluorometric assays in general are not affected by turbidity and, therefore, permit direct analysis of product formation during an enzyme reaction.

Collaborative Study

Eight collaborators, all familiar with the examination of dairy products for ALP activity, agreed to collaborate on this project. Among the collaborators were university, state regulatory, and private industry laboratories. The study of fluid milk ALP was conducted in 3 phases. In phase 1, each laboratory received reagents and a dedicated bench-top fluorometer along with on-site instructions in their use. In phase 2, the collaborators assayed 6 practice pool samples containing known and unknown (to the collaborator) amounts of mixed herd raw milk. No laboratory was allowed to proceed until ability to perform the new method was demonstrated in phase 2.

Phase 3 was the main study. Each laboratory received 4 levels of 4 dairy products containing added mixed herd raw milk as blind duplicates and was asked to assay each vial in duplicate for a total of 4 assays per level. Collaborators assayed all samples by both the fluorometric method and the AOAC method, 16.121-16.122.

Phase 1—Reagents and Supplies

All necessary reagents and supplies for the AOAC method were supplied to each collaborator. Phenol-free phenyl phosphate, neutralized butanol, carbonate buffer, color developer reagent, and phenol standards were prepared from single lots of stock in the Associate Referee's laboratory.

Phase 2—Samples and Data

Six pool samples were prepared as practice samples at 0 and approximately 0.05, 0.1, and 0.5% (v/v) of mixed herd raw milk.

Received for publication March 8, 1990.

This report was presented at the 103rd AOAC Annual International Meeting, September 25-28, 1989, at St. Louis, MO.

The report has been evaluated by the General Referee and the Committee Statistician and reviewed by the Committee on Foods I. The method was approved interim official first action by the Chairman of the Official Methods Board and was adopted official first action at the 104th AOAC Annual International Meeting, September 9-13, 1990, at New Orleans, LA. Association actions will be published in "Changes in Official Methods of Analysis" (1991) *J. Assoc. Off. Anal. Chem.* 74, January/February issue.

raw milk in commercial, homogenized, pasteurized, whole milk. Two mL aliquots of the prepared pools were transferred to plastic screw-cap tubes, frozen at -10°C , and shipped to the collaborators on dry ice by next day air courier. Samples 1 and 2 were blind duplicates of whole, pasteurized whole milk to which no raw milk was added. Samples 4 and 5 were also blind duplicates, and each contained approximately 0.1% (v/v) fresh mixed herd raw milk. Samples 3 and 6 contained 0.05 and 0.5% raw milk, respectively. Collaborators were requested to run the samples in duplicate and to report their results. All practice samples were assayed in the Associate Referee's laboratory by the proposed fluorometric method prior to shipment.

Overall means (mU/L) and standard deviations for the collaborating laboratories for phase 2 samples were as follows (data for the Associate Referee's assays in parentheses): samples 1 and 2, 25.4 ± 5.3 and 28.8 ± 6.5 , respectively (23.4 ± 7.7); samples 4 and 5, 585.3 ± 28.5 and 567.0 ± 74.6 , respectively (563.7 ± 11.9); sample 3, 306.1 ± 20.3 (290.3 ± 5.9); and sample 6, 2758.5 ± 128.6 (2793.7 ± 11.8). Recoveries by the collaborators for the 6 samples ranged from 98.7 to 123.0%.

Phase 3 Samples

Samples for the main phase of the study were prepared in a similar manner to those for phase 2. Fresh mixed herd raw milk was added to commercial, pasteurized, homogenized, whole milk; skim milk; chocolate milk (0.4% fat); and half and half cream (11% fat) at 0 and approximately 0.05, 0.1, and 0.2% (v/v) added raw milk. Each of the 8 laboratories received each level of product as blind duplicates and was asked to assay each vial in duplicate for a total of 4 assays per level by each method. Test portions were shipped frozen to the collaborators on dry ice by next day air courier. Collaborators were instructed to maintain the products at -10°C in a freezer until analyzed. Samples were allowed to thaw at room temperature and then were mixed thoroughly before sampling.

Prior to shipment, each pool sample was assayed in the Associate Referee's laboratory by the fluorometric method and by the AOAC phenyl phosphate method, 16.121-16.122. Means (mU/L) and standard deviations of triplicate assays by the fluorometric method at the 4 prepared levels (0, 0.05, 0.1, and 0.2% added raw milk) were: whole milk, 10.8 ± 3.1 , 226.2 ± 1.5 , 468.0 ± 30.6 , 909.4 ± 38.5 ; skim milk, 12.2 ± 2.5 , 241.0 ± 19.3 , 475.0 ± 33.1 , 932.2 ± 57.5 ; chocolate milk, 7.6 ± 6.8 , 264.5 ± 31.6 , 480.9 ± 57.6 , 983.4 ± 77.5 ; cream (half and half), 0.0, 188.9 ± 19.1 , 377.0 ± 32.8 , 694.6 ± 116.4 mU/L.

Alkaline Phosphatase Activity in Fluid Dairy Products

Fluorometric Method

First Action

(Applicable to whole milk, skim milk, and chocolate milk)

Method Performance:

Whole milk

$s_r = 21.7$; $s_R = 34.6$; $\text{RSD}_r = 4.4\%$; $\text{RSD}_R = 7.0\%$

Skim milk

$s_r = 19.2$; $s_R = 31.4$; $\text{RSD}_r = 3.8\%$; $\text{RSD}_R = 6.2\%$

Chocolate milk

A. Principle

Alkaline phosphatase (ALP) activity in fluid dairy products is measured by continuous fluorometric direct kinetic assay. A nonfluorescent aromatic monophosphoric ester substrate undergoes hydrolysis of its phosphate radical and is converted to a highly fluorescent product. ALP activity is measured in mU/L at 38° during 3-min read time. One Unit of ALP is amount of enzyme that catalyzes transformation of 1 micromole of substrate/min/L of sample. Because of the low levels of ALP in finished dairy products, results are reported in milliUnits/L (mU/L).

B. Apparatus

Items (a)-(d) are available as Fluorophos® Test System (Advanced Instruments, Inc. 1000 Highland Ave, Needham Heights, MA 02194).

(a) *Fluorometer*.—Filter fluorometer with thermostatted cuvet holder held at $38 \pm 0.1^{\circ}$ and right angle optics. Excitation 439 nm and emission 560 nm. Fluorescence output is monitored through analog-to-digital converter into programmable calculator with built-in thermal printer for automatic calculation and printing of results. Alternatively, results may be calculated manually as in H.

(b) *Cuvets*.—Disposable, nonfluorescent glass. 12×75 mm, round.

(c) *Pipettors*.—Fixed volume, 2.0 mL reagent dispenser and positive displacement pipettor at 0.075 mL.

(d) *Incubator block*.—20-well dry bath set at 38° for preincubation of substrate.

C. Reagents

Items (a)-(d) are available as Fluorophos ALP Test Kit (Advanced Instruments Inc.).

(a) *Substrate*.—Fluorophos, 36 mg, freeze-dried in 60 mL glass vials. Stable 1 year at 4° . Fluorophos substrate is water-soluble, nonfluorescent aromatic monophosphoric ester, which is stable 1 year when freeze-dried and stored in glass vials. Exercise normal precautions for handling laboratory reagents.

(b) *Substrate diluent*.—Diethanolamine (DEA) buffer, pH 10.0, 2.4M. Stable 1 year at 4° .

(c) *Working substrate*.—Add 1 vial substrate diluent, (b), to 1 vial (36 mg) substrate, (a). Mix well by inversion. Stable 4 weeks at 4° and 8 h at 38° . Sufficient for 30 tests.

(d) *Working calibrators*.—Fluoroyellow® (FY) in DEA buffer. Calibrator A, 0 $\mu\text{M/L}$ of FY; calibrator B, $17.24 \times 10^{-3} \mu\text{M/L}$ of FY; calibrator C, $34.48 \times 10^{-3} \mu\text{M/L}$ of dephosphorylated aromatic fluorescent product of the enzymatic reaction. Stable 1 year at 4° .

D. Sample Preparation

Use positive displacement pipet to take 0.075 mL aliquot from well-mixed portion of fluid dairy product.

E. Calibration

Each type of dairy product being tested requires its own calibration curve. Calibration curves are stable and need only be run when lot of reagents changes. Calibrators and substrate reagent lots are matched and should not be interchanged.

Dispense 2.0 mL of calibrators A, B, and C, each in duplicate, into labeled 12×75 mm cuvettes. Place cuvettes in incubator.

Table 1. Collaborative results for fluorometric determination of ALP activity (mU/L) in whole milk

Coll.	Level ^a			
	0	0.05%	0.1%	0.2%
10	6.1	270.9	449.5	726.7
	6.1	246.3	400.3	628.2
	18.4	221.7	461.9	843.7
	12.3	190.9	418.8	852.2
20	15.4	277.9	509.5	1019.1
	15.4	277.9	501.8	1011.4
	15.4	254.7	486.4	980.5
	15.4	270.2	501.8	1065.5
30	13.6	246.4	499.7	972.5
	6.8	253.3	499.7	958.4
	13.6	253.3	472.3	944.7
	6.8	253.3	458.7	1040.6
40	13.5	270.8	528.1	995.4
	13.5	291.1	541.7	1036.0
	13.5	277.6	534.9	1029.2
	13.5	284.4	507.8	1049.5
50	15.9	263.2	486.6	961.3
	11.9	275.2	510.5	985.2
	7.9	243.3	494.6	981.2
	3.9	267.2	526.5	1005.1
60	6.0	213.1	523.6	956.0
	18.2	207.0	554.1	901.2
	11.9	280.5	501.4	973.0
	11.9	262.6	501.4	883.4
80	10.5	252.9	490.0	980.0
	5.2	252.9	511.0	964.2
	15.8	252.9	490.0	1001.0
	15.8	247.6	479.4	964.2
90	6.2	243.8	487.6	912.8
	6.2	250.0	475.1	881.5
	14.4	267.0	476.3	1104.1
	14.4	281.4	548.4	1118.6
Mean	11.7	256.2	494.6	960.2

^a Amount of added mixed herd raw milk.

0.075 mL (75 μ L) sample of well mixed dairy product. Dairy product need not be prewarmed.

Gently invert all cuvetts to mix contents and return cuvetts to incubator block. Starting with calibrator A, perform following calibration routine. Set fluorometer to zero fluorescence with calibrator A and then read and record amount of fluorescence obtained with calibrators B and C against calibrator A (0 μ M/L). Wipe outside of cuvet with tissue paper before placing in fluorometer.

When calibration is completed, proceed with analysis of samples.

F. Determination

Bring 2.0 mL working substrate in labeled 12 \times 75 mm cuvet to 38° by placing cuvet in dry bath incubator block.

Add 0.075 mL (75 μ L) well mixed sample to substrate. Immediately mix by gentle inversion, wipe outside of cuvet with tissue paper, and place cuvet in fluorometer. Wait 1 min for temperature equilibration, then record rate of increase in fluorescence (F/min) over next 2 min. Record F/min for each sample and use this value to calculate mU/L of ALP.

Table 2. Collaborative results for fluorometric determination of ALP activity (mU/L) in skim milk

Coll.	Level ^a			
	0	0.05%	0.1%	0.2%
10	12.6	272.9	545.9	1079.2
	12.6	279.3	501.5	1066.5
	6.3	304.7	526.9	1022.1
	6.3	260.2	520.5	1053.8
20	6.8	268.5	537.0	1053.4
	13.7	282.2	523.2	1067.1
	20.6	254.7	502.6	984.5
	6.8	261.6	550.8	984.5
30	5.9	257.9	497.9	947.9
	5.9	251.9	491.9	947.9
	11.9	287.9	497.9	1043.8
	5.9	275.9	503.9	1055.8
40	17.9	250.7	519.4	1050.8
	17.9	268.6	513.4	1026.9
	11.9	274.6	537.3	1074.7
	11.9	280.6	549.2	1086.6
50	10.7	268.2	518.6	933.6
	10.7	253.9	490.0	962.2
	14.3	253.9	486.4	919.2
	14.3	250.3	490.0	958.6
60	15.7	257.0	487.8	949.4
	15.0	251.7	445.8	933.7
	20.5	210.5	446.8	883.4
	15.4	195.1	421.1	816.6
80	14.4	260.7	531.1	1013.9
	14.4	270.3	492.4	970.5
	9.6	255.9	521.4	960.8
	14.4	280.0	492.4	956.6
90	11.4	268.6	543.0	1017.4
	17.1	274.3	548.7	1040.2
	5.0	264.0	505.5	971.8
	5.6	247.1	505.5	994.3
Mean	11.9	262.3	507.6	994.6

^a Amount of added mixed herd raw milk.

G. Controls

(a) *Negative control*.—Include a negative control with each batch of samples. Heat 5 mL dairy product to 95° for 1 min, followed by rapid cooling.

(b) *Positive control*.—Include a positive control at or close to decision level with each batch of samples. Add 0.2 mL fresh, mixed herd, raw milk to 100 mL sample that has been heated to 95° for 1 min.

(c) *Interfering substance control*.—Perform interfering substance control test on all dairy products being tested, including flavored milk. When 0.075 mL dairy product is added to 2.0 mL zero calibrator (instead of working substrate) and this sample is run as a test, no ALP activity should be observed during the 2-min measurement period.

(d) *Microbial ALP control*.—If test for ALP is positive, heat sample for 30 min at 62.8°, and retest for ALP. Any residual activity is caused by microbial ALP.

H. Calculations

Table 3. Collaborative results for fluorometric determination of ALP activity (mU/L) in chocolate milk

Coll.	Level ^a			
	0	0.05%	0.1%	0.2%
10	21.8	276.2	566.9	1112.1
	21.8	276.2	530.6	1053.9
	21.8	276.2	545.1	1090.3
	29.0	254.4	596.8	1024.9
20	10.0	267.0	523.8	1027.2
	10.2	267.0	513.6	1027.2
	10.2	308.1	554.6	1119.6
	10.2	277.3	554.6	1098.2
30	8.4	254.3	508.6	1000.3
	8.4	254.3	483.2	991.8
	8.4	271.2	525.6	991.8
	8.0	254.3	534.0	1068.1
40	8.8	284.2	639.5	1137.0
	8.8	293.1	639.5	1145.9
	26.6	284.2	550.7	1092.6
	17.7	293.1	532.9	1092.6
50	4.8	271.5	460.7	969.9
	4.0	247.3	460.7	960.2
	4.8	252.1	480.1	940.8
	4.0	242.4	494.6	955.4
60	0.0	249.7	499.4	1013.5
	0.0	235.0	514.0	998.8
	7.0	276.3	538.5	1006.2
	7.0	262.2	510.2	942.5
80	19.5	247.5	495.1	977.3
	6.0	273.6	521.2	970.3
	6.5	247.5	469.1	983.8
	6.5	241.0	495.1	944.7
90	7.4	251.8	488.8	970.2
	7.0	237.0	466.6	985.0
	0.0	247.1	486.8	973.7
	0.0	239.6	501.8	981.2
Mean	9.8	262.8	521.3	1020.2

^a Amount of added mixed herd raw milk.

brators B and C read against calibrator A set to zero fluorescence on fluorometer.

Record increase in fluorescence of sample as $\Delta F/\text{min}$.

To calculate ALP enzyme activity, mU/L, calculate $\mu\text{moles FY}$ formed per minute by 0.075 mL sample by using fluorescence reading of calibrator B, which contains $3.448 \times 10^{-5} \mu\text{M FY}$.

$\mu\text{M FY}/\text{min}/0.075 \text{ mL}$

$$= [(\Delta F/\text{min}/0.075 \text{ mL sample})/F \text{ of calibrator B}] \times (3.448 \times 10^{-5})$$

To calculate $\mu\text{moles FY}$ formed by 1 L sample, multiply result obtained above by 13333.3, and then multiply that value by 1000 to convert to mU/L. In summary,

ALP activity, mU/L

$$= [(\Delta F/\text{min}/0.075 \text{ mL sample})/F \text{ of calibrator B}] \times 459.7$$

Ref.: JAOAC 73, November/December issue (1990).

Table 4. Collaborative results for fluorometric determination of ALP activity (mU/L) in cream (half and half)

Coll.	Level ^a			
	0	0.05%	0.1%	0.2%
10	6.4	134.9	314.8	571.9
	1.2	128.5	327.7	539.7
	6.4	160.6	340.5	610.4
	6.4	147.7	340.5	616.8
20	9.0	101.8	305.4	444.2
	9.0	120.3	323.9	509.0
	18.5	157.3	397.9	536.7
	27.7	138.8	444.2	573.8
30	7.0	179.6	337.8	558.1
	7.3	154.2	337.8	521.4
	14.6	198.2	403.9	712.3
	14.6	212.9	352.5	903.3
40	7.2	137.1	310.3	505.1
	7.2	137.1	281.4	541.2
	14.4	129.9	281.4	497.9
	7.2	115.4	360.8	519.6
50	4.9	132.5	319.0	520.2
	4.0	152.1	289.5	500.6
	9.8	171.7	279.7	677.2
	4.9	157.0	274.8	652.7
60	6.3	195.6	359.7	845.7
	6.3	201.9	429.1	858.3
	6.5	209.6	412.7	917.2
	6.5	235.8	465.1	818.9
80	0.0	145.2	262.5	497.0
	0.0	167.5	268.0	474.7
	11.1	150.8	279.2	541.7
	5.5	145.2	284.8	558.5
90	7.0	118.9	267.6	639.3
	7.4	170.9	267.6	602.1
	6.0	129.6	265.8	635.4
	6.4	155.6	278.8	629.0
Mean	8.0	156.0	327.0	610.3

^a Amount of added mixed herd raw milk.

study for the pool milk samples by the fluorometric procedure; Table 6 gives the collaborative results for the AOAC method.

Table 7 lists the means and statistical summary of the results obtained in the collaborating laboratories on the 16 different pools of milk. The major focus of the study was to examine the reproducibility of the fluorometric method, especially at the 0.1% (v/v) or 1.0 μg phenol/mL ALP activity level. Reproducibility among laboratories (relative standard deviation, RSD_R) for the whole milk, skim milk, and chocolate milk samples at this level ranged between 7.0 and 8.8%, which is acceptable. The greater variation (18.1%) for cream samples at this level may be attributable to the difficulties encountered with the cream pool, which contained 11% fat content. The thawed samples did not become adequately resuspended into a homogenous sample. ALP is known to adhere to fat globules, and this may account for the lower recovery in the cream samples.

A total of 8 pairs of test results for the AOAC ALP method

Table 5. Summary of collaborative results for fluorometric determination of ALP activity (mU/L) in fluid dairy products^a

Level, % ^b	Material mean	Laboratory mean							
		10	20	30	40	50	60	80	90
Whole milk									
0	11.7	10.7	15.4	10.2	13.5	9.9	12.0	11.8	10.3
0.05	256.2	232.4	251.3	251.5	266.2	262.2	240.8	251.5	260.5
0.1	494.6	432.6	499.8	482.6	528.1	504.5	520.1	492.6	496.8
0.2	960.2	762.7	1019.1	979.0	1027.5	983.2	928.4	977.3	1004.2
Skim milk									
0	11.9	9.4	11.9	7.4	14.9	12.5	16.6	13.2	9.7
0.05	262.3	279.2	266.7	268.4	268.6	256.5	228.5	266.7	263.5
0.1	507.6	523.7	528.4	497.9	529.8	496.2	450.3	509.3	525.6
0.2	994.6	1055.4	1022.3	998.8	1059.7	943.4	895.7	975.4	1005.9
Chocolate milk									
0	9.8	23.6	10.1	8.3	15.4	4.4	3.5	9.6	3.6
0.05	262.8	270.7	279.8	258.5	288.6	253.3	255.8	252.4	243.8
0.1	521.3	411.6	536.6	512.8	590.6	474.0	515.5	495.1	486.0
0.2	1020.2	1070.3	1068.0	1013.0	1117.0	956.5	990.2	969.1	977.5
Cream (half and half)									
0	8.0	5.1	16.0	10.8	9.0	5.9	6.4	4.1	6.7
0.05	156.0	142.9	129.5	186.2	129.8	153.3	210.7	152.1	143.7
0.1	327.0	330.8	367.8	358.0	308.4	290.7	416.6	273.6	269.9
0.2	610.3	584.7	515.9	673.7	515.9	587.6	860.0	517.9	626.4

^a Collaborator assayed each blind duplicate pair in duplicate at each level.

^b Amount of added fat.

^a Collaborator assayed each blind duplicate pair in duplicate at each level.^b Amount of added mixed herd raw milk.Table 6. Collaborative results for determination of alkaline phosphatase as μg phenol/mL in fluid dairy products by AOAC method 16.121-16.122

Level, % ^a	Laboratory								Material mean
	10	20	30	40	50	60	80	90	
Whole milk									
0	0.00	0.00	0.03	0.00	0.30	0.00	0.20	0.10	0.11
	0.15	0.00	0.00	0.00	0.30	0.00	0.25	0.06	
	1.60 ^b	0.00	0.00	0.00	0.50	0.00	0.20	0.00	
	1.08 ^b	0.36	0.03	0.00	0.50	0.00	0.25	0.00	
0.05	0.00	1.60	0.74	0.55	0.70	0.56	1.20	0.76	0.83
	2.10	1.46	0.72	0.70	0.60	0.00 ^b	1.00	0.86	
	2.00 ^b	1.20	0.65	0.75	0.80	0.80	1.00	0.52	
	3.00 ^b	1.00	0.71	0.90	0.80	0.26	0.90	0.35	
0.1	1.72	2.10	1.19	1.25	ND ^c	0.86	1.90	1.20	1.46
	1.00	2.00	1.15	1.40	1.30	0.00 ^b	1.90	1.24	
	0.35 ^b	2.46	1.45	1.60	1.30	1.36	1.50	1.28	
	0.50 ^b	1.90	1.46	1.72	ND	0.52	1.55	1.28	
0.2	3.00	5.00	2.38	2.50	1.30	0.00 ^b	3.50	2.50	2.70
	3.30	5.20	2.38	2.85	1.00	ND	3.50	2.54	
	2.40 ^b	2.90	2.64	3.20	2.20	1.32	3.10	2.16	
	3.70 ^b	2.80	2.64	3.30	2.20	1.48	2.80	2.00	
Skim milk									
0	1.08	0.00	0.00	0.14	0.80	0.00	0.05	0.00	0.07
	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00	
	2.00 ^b	0.00	0.00	0.00	0.00	0.00	0.02	0.00	
	1.00 ^b	0.00	0.00	0.00	0.00	0.00	0.05	0.00	
0.05	0.67	1.30	0.47	0.35	0.80	0.00	0.70	0.52	0.07
	0.92	1.34	0.36	0.44	0.70	0.60	0.90	0.52	
	1.01 ^b	0.60	0.53	0.50	0.60	0.01	0.80	0.46	
	1.01 ^b	0.56	0.51	0.65	0.60	0.30	0.70	0.46	

Table 6. Continued

Table 6. Continued									
Level, % ^a	Laboratory								Material mean
	10	20	30	40	50	60	80	90	
0.1	1.18	2.16	0.82	0.75	0.60	0.58	1.40	1.28	1.02
	0.82	1.88	0.84	1.00	0.40	0.00	1.40	0.98	
	0.00 ^b	1.44	1.04	1.15	1.00	0.18	1.30	0.94	
	0.10 ^b	1.86	1.01	1.30	1.00	0.04	1.20	1.00	
0.2	1.74	4.56	1.75	1.90	2.20	1.70	2.60	2.00	2.14
	1.74	4.00	1.75	2.27	2.20	1.00	2.45	2.08	
	0.70 ^b	2.62	1.89	2.60	1.60	0.52	2.30	ND	
	1.50 ^b	3.10	1.84	2.90	1.60	0.56	2.35	ND	
Chocolate milk									
0	0.00	0.00	0.00	0.00	0.00	0.00	0.20	0.02	0.07
	0.00	0.00	0.00	0.10	0.00	0.00	0.25	0.10	
	0.00	0.36	0.10	0.00	0.00	0.00	0.30	0.10	
	0.00	0.00	0.20	0.10	0.00	0.00	0.30	0.00	
0.05	0.20	0.60	0.17	0.44	0.00	0.10	0.60	0.40	0.38
	0.86	0.40	0.21	0.54	0.00	0.28	0.70	0.40	
	0.00 ^b	0.50	0.30	0.72	0.40	0.04	0.55	0.14	
	0.90 ^b	0.60	0.37	0.72	0.40	0.04	0.70	0.12	
0.1	0.80	1.30	0.47	0.75	0.00 ^b	0.44	0.85	0.48	0.78
	0.00 ^b	1.10	0.24	1.12	1.10	0.26	0.95	0.70	
	0.05 ^b	1.02	0.62	1.50	0.40	0.00 ^b	0.90	0.66	
	ND	0.90	0.67	ND	0.40	0.00 ^b	1.30	0.50	
0.2	2.30	2.84	0.84	1.20	0.00 ^b	0.66	2.05	1.40	1.40
	0.00 ^b	2.84	0.97	1.44	0.00 ^b	0.86	2.05	1.90	
	0.00 ^b	1.60	0.85	2.16	1.20	0.34	1.75	1.24	
	0.00 ^b	1.00	0.78	ND	0.80	0.34	1.90	0.98	
Cream (half and half)									
0	0.00	0.00	0.07	0.12	0.00	0.36	0.30	0.10	0.20
	0.00	0.00	0.09	0.12	0.00	0.48	0.30	0.00	
	0.00	0.20	0.18	0.84	0.30	0.30	0.40	0.00	
	0.00	0.00	0.18	0.55	0.70	0.14	0.70	0.00	
0.05	0.15	0.34	0.94	0.80	0.70	0.40	0.90	0.40	0.75
	0.10	0.40	0.87	0.70	0.80	0.70	0.90	0.02 ^b	
	0.00 ^b	1.28	0.95	1.40	0.50	0.00 ^b	1.00	0.52	
	0.04 ^b	1.00	0.89	0.97	0.50	0.00 ^b	1.50	0.60	
0.1	1.25	1.88	1.36	1.35	1.20	1.36	0.06	1.20	1.33
	0.90	1.06	1.33	1.42	1.20	1.50	1.50	1.28	
	0.00 ^b	1.80	1.57	2.10	0.90	0.86	1.65	1.20	
	1.40 ^b	1.74	1.45	1.90	0.90	0.74	2.05	1.24	
0.2	2.30	4.80	2.84	2.55	2.00	2.20	3.05	1.80	2.68
	0.00 ^b	2.16	2.74	2.52	2.20	2.40	2.90	2.00	
	0.52 ^b	3.20	2.99	3.56	1.50	1.90	3.05	2.00	
	1.12 ^b	4.26	3.08	2.95	1.50	0.09 ^b	3.00	3.56	
Amount of added mixed herd raw milk.									

^a Amount of added mixed herd raw milk.^b Technical outliers by laboratory not included in statistical analysis by Grubbs test.^c ND = no data submitted.

Grubbs tests. The statistical outliers for Laboratory 60 appear to have been caused by the laboratory incorrectly running and reporting the wrong samples.

The mean values for the blank samples (level 1) in the 4 dairy products tested ranged from 8.0 to 11.9 mU/L with the fluorometric assay. At levels of contamination equivalent to 0.4 to 0.8 µg phenol/mL, the fluorometric method gave a range of 156.0 to 262.8 mU/L, which indicates good discrimination at approximately half the upper limit of acceptability for fluid dairy products. At the cutoff value of approximately 1.0 mU/L, the fluorometric method gave a

mean value of 494.6 mU/L for the whole milk, 507.6 for the skim milk, and 521.3 for the chocolate milk. The cream samples at this level gave a mean of 327.0 mU/L. In all 4 products, response was linear for the fluorometric assay with increasing concentration of raw milk from 0.05 to 0.2% (v/v) (Figure 1).

Summary

Repeatability (within-laboratory relative standard deviation RSD) at the 0.1% level was 1.0% for whole milk, 1.0% for skim milk, 1.0% for chocolate milk, and 1.0% for cream.

Table 7. Statistical summary^a of collaborative results for determination of alkaline phosphatase in fluid dairy products by fluorometric method and AOAC method 16.121-16.122

Product	Level	Fluorometric method, ALP activity, mU/L					AOAC method, ALP value, μ g phenol/mL				
		Mean	s_r	RSD _r , %	s_R	RSD _R , %	Mean	s_r	RSD _r , %	s_R	RSD _R , %
White milk	0	11.7	4.3	36.9	4.3	36.9	0.11	0.08	76.3	0.16	146.3
	0.05	256.2	19.9	7.8	23.3	9.1	0.83	0.37	45.4	0.41	49.7
	0.1	494.6	21.7	4.4	34.6	7.0	1.46	0.24	16.7	0.42	29.6
	0.2	960.2	64.3	6.7	101.7	10.6	2.70	0.60	23.1	0.97	37.6
Skim milk	0	11.9	3.9	33.2	4.5	38.3	0.07	0.21	219.0	0.24	240.0
	0.05	262.3	16.5	6.3	20.7	7.9	0.60	0.20	33.9	0.29	47.7
	0.1	507.6	19.2	3.8	31.4	6.2	1.02	0.21	21.4	0.51	50.6
	0.2	994.6	38.7	3.9	64.6	6.5	2.14	0.45	21.7	0.88	42.0
Chocolate milk	0	9.8	4.4	45.9	7.8	80.9	0.07	0.07	77.0	0.10	109.0
	0.05	262.8	12.8	4.9	18.9	7.2	0.38	0.17	42.9	0.24	60.0
	0.1	521.3	27.6	5.3	45.8	8.8	0.78	0.24	31.8	0.33	44.2
	0.2	1020.2	30.6	3.0	64.2	6.3	1.40	0.46	31.7	0.72	49.3
Cream (half and half)	0	8.0	4.3	54.7	5.4	67.8	0.20	0.19	84.3	0.24	104.3
	0.05	156.0	18.5	11.9	32.6	20.9	0.75	0.26	37.6	0.35	51.7
	0.1	327.0	34.0	10.4	59.1	18.1	1.33	0.40	30.9	0.42	32.1
	0.2	610.3	76.2	12.5	133.6	21.9	2.68	0.60	23.0	0.79	30.0

^a s_r = repeatability standard deviation; s_R = reproducibility standard deviation; RSD_r = repeatability (within-laboratory) relative standard deviation; RSD_R = reproducibility (among-laboratories) relative standard deviation.

^b Amount of added mixed herd raw milk.

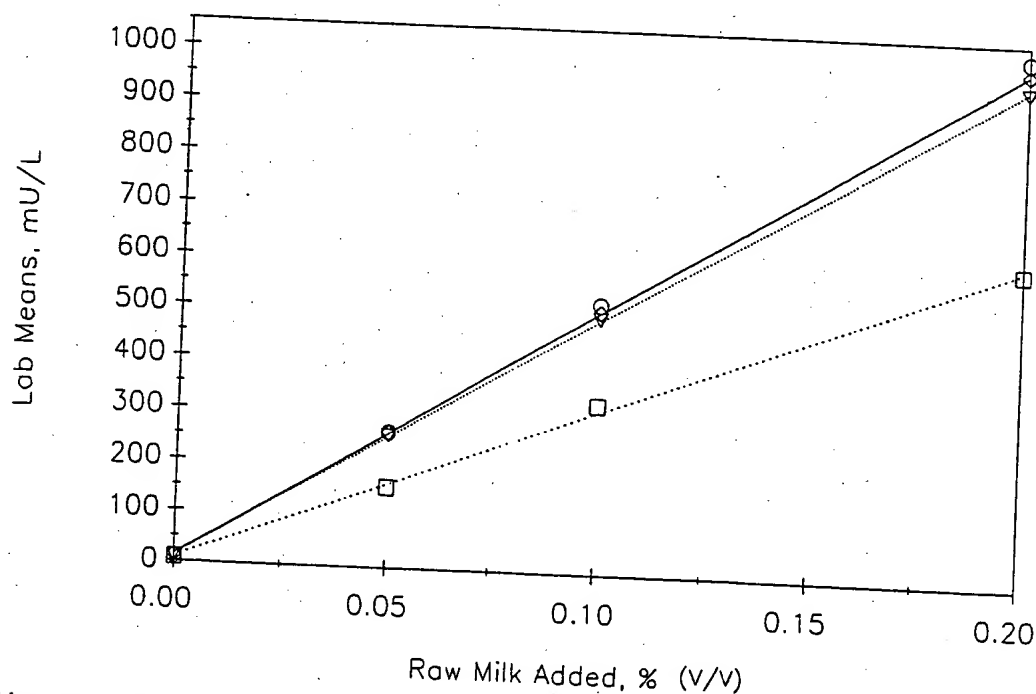


Figure 1. Linearity of response for Fluorophos alkaline phosphatase test in fluid milks: ∇ = whole milk, \diamond = skim milk, \circ = chocolate milk, \square = cream (half and half).

provement in repeatability compared to the AOAC method which gave a range of 16.7-31.8% for the same samples of whole milk, skim milk, and chocolate milk.

Reproducibility (among-laboratories relative standard deviation, RSD_R) for the fluorometric method for the same 3 products was 6.2-8.8% compared to 29.6-50.6% for the AOAC method. The significant reduction in analytical steps from over 5 in the AOAC method to 1 in the fluorometric assay may help account for this improvement. Collaborators

commented on the difficulty of adequately resuspending the frozen cream (half and half) samples. Further work with cream will require using freshly prepared samples.

Recommendations

The Associate Referee recommends (1) that the fluorometric ALP method be adopted official first action as a new method for measurement of ALP in whole milk, skim milk, and chocolate milk; and (2) that further collaborative studies

be conducted to assess the suitability of this method for other dairy products including cheese, whey, and cream.

Acknowledgments

The cooperation of the following collaborators is gratefully acknowledged:

R. L. Bradley, Jr, M. Lundberg, and J. Beyer, University of Wisconsin, Madison, WI

D. Elliott and B. Thornhill, Florida Dept of Agriculture, Winter Haven, FL

L. Hensel, Mid-America Dairymen, Inc., Winsted, MN
J. Jaworski, R. Cyr, and L. Justis, Vermont Dept of Agriculture, Montpelier, VT

D. H. Kleyn and L. Lengyel, Rutgers University, New Brunswick, NJ

R. K. Stuckey, T. Cronau, J. Siebodnik, and J. Scott, Indiana State Board of Health, Indianapolis, IN

T. Way, C. Mayfield, and C. Strayer, Applied Microbiology Services, Inc., College Station, TX

H. M. Wehr and B. Burwell, Oregon Dept of Agriculture, Salem, OR

REFERENCES

- (1) Kay, H. D., & Graham, W. R. (1935) *J. Dairy Res.* 6, 191-203
- (2) *Official Methods of Analysis* (1984) 14th Ed. and (1990) 15th Ed., AOAC, Arlington, VA
- (3) Rocco, R. M. (1990) *J. Food Prot.* 53, 588-591

Kjeldahl Method for Determination of Total Nitrogen Content of Milk: Collaborative Study

DAVID M. BARBANO and JENNY L. CLARK

Cornell University, Department of Food Science, Ithaca, NY 14853

CHAPMAN E. DUNHAM and J. RICHARD FLEMING¹

Texas Milk Market, PO Box 110939, Carrollton, TX 75011

Collaborating Laboratories: Cornell University and Northeast Dairy Herd Improvement Cooperative; and laboratories operated by or under contract to the following Federal Milk Markets: Chicago Regional; Eastern Ohio/Western Pennsylvania; Greater Kansas City; New England; Texas; Upper Midwest

A macro-Kjeldahl procedure using a copper catalyst for determination of milk total nitrogen was developed for both traditional and block digester/steam distiller equipment, and the performance was evaluated by collaborative study. In the first trial of the collaborative study, 9 pairs of blind duplicate milk samples were analyzed for total nitrogen and total nitrogen was converted to "protein" by using a factor of 6.38. Protein content of milk samples ranged from 3.086 to 3.610%. In the first trial, s_R and R values for the block digestors were influenced significantly by protein concentration; s_R and R values were not influenced by protein concentration for traditional equipment. It was hypothesized that total digestion time for some block digestors in the first trial was not sufficient for high protein milk samples. Thus, a second trial was undertaken with boiling time after clearing increased by 0.5 h. In the second trial, none of the parameters for reproducibility with either type of equipment were influenced by protein concentration. It was concluded that laboratory-to-laboratory differences in line voltage may require different total digestion times in different laboratories, particularly those using block digestors. The Kjeldahl method using a copper catalyst and either traditional or block digester equipment for determination of milk total nitrogen has been adopted official first action by AOAC to replace method 920.105.

protein determination that accurately measures protein. Infrared milk analyzers, now in commercial use by the dairy industry, can be calibrated to predict the protein content of milk [972.16, 15th Ed. (1)] based on infrared light absorbance at 6.465 μm wavelength by the N-H bonds within the protein. Data from an accurate reference method for milk protein determination is necessary for proper calibration of infrared milk analyzers.

The Kjeldahl method measures nitrogen and from the nitrogen content of a sample the protein content can be estimated. The Kjeldahl method has been widely studied (2-14). Many researchers have attempted to substitute reagents (3-7, 10, 11), vary reagent quantities (5, 8, 9, 12, 13), and optimize digestion parameters (8, 9, 12-14) to improve the test accuracy, decrease testing time, and eliminate hazardous chemicals (e.g., mercury) that have a detrimental impact on the environment.

The Kjeldahl method uses an acid digestion to release bound organic nitrogen and retain it as ammonium sulfate

Received for publication February 27, 1990.

This report was presented at the 103rd AOAC Annual International Meeting, September 25-28, 1989, at St. Louis, MO.

The report has been evaluated by the General Referee and the Committee Statistician and reviewed by the Committee on Foods I. The method was approved interim official first action by the Chairman of the Official Methods Board and was adopted official first action at the 104th AOAC Annual International Meeting, September 9-13, 1990, at New Orleans, LA. Association actions will be published in "Changes in Official Methods of Analysis" (1991) *J. Assoc. Off. Anal. Chem.* 74, January/February issue.

¹J. Richard Fleming is Chairman, Test Procedures Committee.

Dairy farmers in some regions of the United States are paid on the basis of both the fat and protein contents of their milk or receive bonus payments for high milk protein content. Thus, it is very important to have a reference method for

Prostatic Acid Phosphatase Assay with Self-Indicating Substrate 2,6-Dichloro-4-acetylphenyl Phosphate

Susumu Osawa,^{1,3} Shinji Iida,¹ Hiroshi Yonemitsu,¹ Katsumasa Kuroiwa,² Katsuhiro Katayama,² and Takeshi Nagasawa²

We characterized six self-indicating substrates, synthesized as the derivative compounds of acetylphenyl phosphate, for serum prostatic acid phosphatase (PAP) activity. One of the substrates, 2,6-dichloro-4-acetylphenyl phosphate (DCAPP), is superior to others in terms of stability, affinity, and low K_m for PAP. The hydrolyzed product, 2,6-dichloro-4-acetylphenol (DCAP), has a maximum absorption at 334.2 nm, a pK_a of 4.15, and a molar absorptivity at 340 nm of $21\,490\text{ L}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$ in citrate-HCl buffer, pH 5.4. PAP activity was assessed by subtracting tartaric acid-inhibited acid phosphatase activity from total acid phosphatase activity. Our assay system involving DCAPP is a unique kinetic method that shows good reproducibility, wide analytical dynamic range, and high specificity for PAP. Moreover, it is easily adaptable to automated analyzers because the product, DCAP, can be monitored at 340 nm.

Indexing Terms: enzyme kinetics/enzyme immunoassay

Many methods for the determination of serum acid phosphatase (ACP; EC 3.1.3.2) activity have been reported, but most of them were not adaptable to automated analyzers (1-3).⁴ Only the kinetic method of Hillmann and its modifications (4-6), which basically utilize 1-naphthyl phosphate (1-NA) and diazo dye Fast Red TR, have been adapted to automated analyzers. Although 1-NA was superior to other substrates in terms of specificity, its color reaction was subject to interference by bilirubin (7). Another kinetic method involving a self-indicating substrate, 2,6-dichloro-4-nitrophenyl phosphate (DCNPP), has been reported (8). The hydrolyzed product of DCNPP, 2,6-dichloro-4-nitrophenol (DCNP), shows stronger 400-nm absorption at pH < 9 than does 4-nitrophenol and can be monitored by automated analyzers at pH 5.4. However, this method also has several disadvantages; e.g., serum albumin quantitatively accelerates the rate of hydrolysis of DCNPP to DCNP (9), and hemoglobin, denatured

in acid solution, influences spectrophotometric measurement at 405 or 415 nm.

To develop an assay that does not have these drawbacks, we focused on six synthesized derivative compounds of acetylphenyl phosphate (10), and found that 2,6-dichloro-4-acetylphenyl phosphate (DCAPP) had advantages of stability, affinity, and K_m for prostatic acid phosphatase (PAP).

In this study, we describe the characteristics of a new assay and its application to automated analyzers.

Materials and Methods

Apparatus

Spectra of substrates and their products were analyzed with a Hitachi U-3200 spectrophotometer (Hitachi, Tokyo, Japan) and enzyme activity was measured with a Hitachi 7050 automated analyzer. The enzyme immunoassay (EIA) was done with an IB-500 analyzer (Toyobo, Tokyo, Japan).

Reagents

Substrates [DCAPP; 2,6-dichloro-4-propionylphenyl phosphate; 2,6-dichloro-4-(2-butyryl)phenyl phosphate; 2,6-dichloro-4-(1-butyryl)phenyl phosphate; 2,6-difluoro-4-acetylphenyl phosphate (DFAPP), 2,6-dibromo-4-acetylphenyl phosphate (DBAPP)] and their hydrolyzed products were obtained from Nitto Boseki (Fukushima, Japan). Bovine serum albumin and human PAP were obtained from Sigma Chemical Co. (St. Louis, MO). Assay kits for DCNPP, 1-NA, and PAP EIA were purchased from Ono (Osaka, Japan), Boehringer Mannheim (Tokyo, Japan), and Dainabot (Tokyo, Japan), respectively. All other reagents were analytical grade and purchased from Wako Pure Chemicals (Osaka, Japan). In measurement of total ACP (T-ACP) activity, reagent 1 consists of citrate buffer (0.1 mol/L sodium citrate-HCl, pH 5.4, and 5.0 g/L bovine serum albumin, which gives a final concentration of 3.8 g/L in assay mixture) and reagent 2 contains substrate (6.0 mmol/L DCAPP in 0.01 mol/L sodium citrate-HCl, pH 3.0). In measurement of tartaric acid-inhibited ACP (TIAP) activity, citrate-HCl buffer containing 26 mmol/L L-(+)-tartaric acid was used instead of reagent 1. These reagents were stable for at least 1 year at 4°C.

Samples

PAP in serum was stabilized by adding 10 μL of 3.3 mol/L acetic acid per 1.0 mL of serum immediately after serum was separated from clotted blood. Pre-treated sera were stored at -20°C until use.

¹ Department of Laboratory Medicine, Chiba University Hospital, 1-8-1 Inohana, Chuo-ku, Chiba City, Chiba 260, Japan.

² Nitto Boseki, Ltd., Biochemical Laboratory, Fukushima, Koriyama City, Fukushima 963, Japan.

³ Author for correspondence. Fax 043-226-2367.

⁴ Nonstandard abbreviations: ACP, acid phosphatase; PAP, prostatic acid phosphatase; T-ACP, total acid phosphatase; TIAP, tartaric acid-inhibited acid phosphatase; DCAPP, 2,6-dichloro-4-acetylphenyl phosphate; DFAPP, 2,6-difluoro-4-acetylphenyl phosphate; DBAPP, 2,6-dibromo-4-acetylphenyl phosphate; DCAP, 2,6-dichloro-4-acetylphenol; 1-NA, 1-naphthyl phosphate; DCNPP, 2,6-dichloro-4-nitrophenyl phosphate; DCNP, 2,6-dichloro-4-nitrophenol; and EIA, enzyme immunoassay.

Received March 28, 1994; accepted October 25, 1994.

Tissue Extracts

To assess K_m values of ACP from various organs, we prepared ACP from tissue extracts by homogenization and centrifugation (1400g, 10 min, 5°C) in 0.1 mol/L citrate-HCl buffer (pH 5.4) (11).

PAP Assay

In experiments comparing substrates, we pipetted 100 μ L of sample, mixed it with 2 mL of buffer solution, incubated the mixture for 3 min to reach 37°C, and started monitoring the reaction rate at 340 nm immediately after adding 500 μ L of substrate solution. The final pH of the mixture was 5.4. We used the Hitachi 7050 automated analyzer in other experiments, mixing 20 μ L of sample with 400 μ L of buffer solution and then adding 100 μ L of substrate solution to start the reaction. We monitored the reaction in rate mode from 140 to 240 s at 340 nm and calculated the enzyme activity from $\Delta A/\text{min}$. We estimated the PAP activity by subtracting TIAP activity from T-ACP activity; we investigated the inhibitory effects of L-tartaric acid on PAP activity in crude extract from various organs.

Characterization of Substrates and Their Products

To characterize the six synthesized substrates and their products, we determined their pH optimum, non-enzymatic hydrolysis, K_m , and molar extinction coefficient.

Nonenzymatic hydrolysis was assessed by measuring the 340-nm absorbance of the substrate solution (final concentration 1.15 mmol/L) at 37°C against a simple buffer solution.

K_m was calculated by a Lineweaver-Burk plot. To obtain the K_m of ACP from various organs, we used eight concentrations of DCAPP between 0.01 and 0.5 mmol/L and measured each point three times.

The apparent ϵ was determined by using the Hitachi U-3200 spectrophotometer and a Hitachi automated analyzer.

Interferences

Besides examining interferences of various compounds with PAP activity, we also investigated the effects of various abnormal sera on the spectrophotometry itself, not on the enzyme activity. The sera tested were hemolytic ($n = 10$; maximum concentration 1000 mg/L as hemoglobin), icteric ($n = 13$; maximum concentration 20 mg/L as total bilirubin), and lipemic ($n = 5$; turbid appearance). In this experiment we omitted substrate from reagent 2.

Comparison of Methods

To compare the present method (applied to automated analyzers) with the DCNPP method, the 1-NA method, and the PAP EIA method, we used several stabilized human sera. The enzymatic methods were run on the Hitachi 7050 analyzer and the PAP EIA method on the IB-500 analyzer according to each manufacturer's instructions.

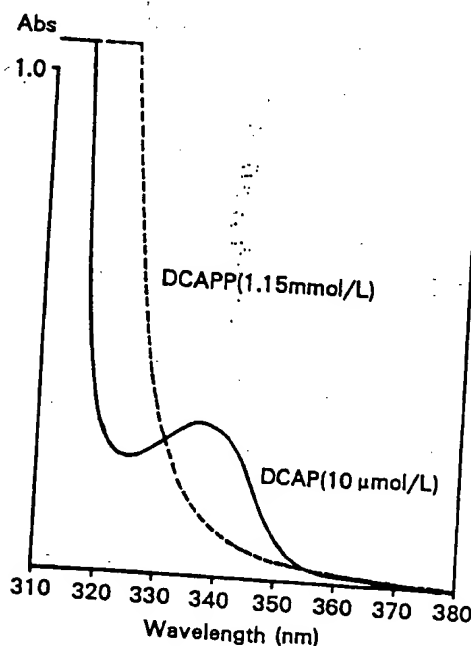


Fig. 1. Absorption curves of DCAPP (---) and DCAP (—). Curves were obtained by scanning the solution of DCAPP or DCAP (1.15 mmol/L and 10 μ mol/L in 0.1 mol/L citrate-HCl buffer, pH 5.4, respectively) against the buffer solution.

Results

Characterization of Substrates and Their Products

The optimum pH for DFAPP and DBAPP was 5.6 and 5.8, respectively; that for the other four substrates was 6.0.

Nonenzymatic hydrolysis accounted for between 0.5×10^{-3} and 3.8×10^{-3} A/min at pH 5.4.

The K_m of the six substrates ranged from 0.147 to 0.233 mmol/L. DCAPP had the smallest K_m value and the highest velocity in the PAP assay, and showed satisfactory stability as a substrate. Therefore, we chose DCAPP as the substrate for PAP activity measurement. The absorption curves of DCAPP and DCAP are shown in Fig. 1.

DCAP had a maximum absorption at 334.2 nm, a pK_a of 4.15, and a molar absorptivity of $21490 \text{ L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ at 340 nm in citrate-HCl buffer (pH 5.4) containing 3.8 g/L bovine serum albumin. The pK_a of DCAP was 4.6 in 0.1 mol/L citrate-HCl buffer, but the apparent pK_a shifted to 4.15 and the ϵ increased from 15200 to $21490 \text{ L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ when bovine serum albumin was added to the buffer solution at a final concentration of 3.8 g/L.

Optimization of Variables for PAP Assay

Buffer solution. After we had examined various buffer solutions for substrates, we selected citrate-HCl buffer because of its stabilizing effect on substrate, absorbance of DCAP, tartaric inhibition of DCAPP, and pK_a . The pH optimum for the PAP-catalyzed hydrolysis of DCAPP in citrate-HCl buffer was ~ 5.6 , with activity $\geq 95\%$ maximum over the range 4.8–6.2. We selected pH 5.4 because the ionized DCAP dissociates nearly 100% at that pH and autohydrolysis of the substrate was less marked at pH 5.4 than at 6.0. The buffer

Table 1. K_m for human ACP from various organs with DCAPP as substrate.

Origin of enzyme	K_m (10^{-3} mol/L)
Prostate	0.147
Kidney	1.250
Liver	1.429
Heart	0.800
Lung	1.667
Bone	2.500
Erythrocytes	2.857
Leukocytes	2.000
Platelets	1.667

Table 2. Results of interference study.

Substances tested	Maximum conc with no interference mmol/L (except as indicated)
Ascorbic acid	2.5
Bovine serum albumin	50.0 (g/L)
Ditaurobilirubin	0.23
Bilirubin	0.3
Glutathione (reduced)	1.5
Glucose	25.0
Hemoglobin	4.5 (g/L)
Uric acid	1.2
CaCl ₂	50.0
FeCl ₂	0.25
NaCl	600.0
Sodium citrate	35.0
EDTA-2Na	5.0
Oxalic acid	30.0
Sodium heparin	200.0 (mg/L)

concentration was set to 0.1 mol/L because PAP activity was maximum.

K_m for human ACP in various organs. K_m values for ACP from various human organs in 0.1 mol/L citrate-HCl buffer (pH 5.4) containing 3.8 g/L bovine serum albumin is summarized in Table 1. The K_m of PAP (1.4×10^{-4} mol/L) was 0.1 that of other organ ACPs, and DCAPP showed stronger affinity for PAP than for other organ ACPs. The final concentration of DCAPP in the PAP assay was 1.15×10^{-3} mol/L, based on maximum velocity (V_{max}).

Evaluation of the Present Method by Automated Analyzers

Molar absorptivity (ϵ). As described above, the apparent ϵ for DCAP was determined to be $21\,490 \text{ L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ at 340 nm on the Hitachi U-3200 spectrophotometer. With the Hitachi 7050 automated analyzer, ϵ was $19\,950 \text{ L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ at 340 nm, and the calculated K-factor was 1295.

Analytical range. The detection limit of the PAP assay was calculated according to Miller and Miller (12). The mean value obtained from 30 measurements of a blank (isotonic saline) was 0.83 nkat/L (SD 1.7 nkat/L), and the detection limit was 5.0 nkat/L. The upper limit of linearity with PAP was 2167 nkat/L at 37°C.

Precision. Within-run imprecision was evaluated with three different concentrations of serum samples assayed 20 times each. The means and CVs for low, medium, and high concentrations of PAP were 17.2, 150, and 415 nkat/L and 8.30%, 1.19%, and 0.48%, respectively. Between-run imprecision was evaluated with two stabilized pooled serum samples. The means and CVs were 173 and 723 nkat/L and 1.20% and 1.23% ($n = 20$), respectively.

Recovery. Analytical recovery was assessed with Precinorm-E control serum (151 nkat/L) with addition of three concentrated PAP solutions (92, 385, 992 nkat/L). The recovery was between 103% and 104%.

Interferences. Substances tested did not interfere with measured PAP activity. Spectrophotometrically, the interfering effects of abnormal sera tested were negligible at those concentrations (Table 2).

Methods Comparison

Correlation coefficients between the present method (y) and the 1-NA method, the DCNPP method, and the PAP EIA method were 0.999 ($y = 0.96x + 0.2$, $n = 9$), 0.995 ($y = 0.80x - 7.7$, $n = 98$), and 0.986 ($y = 0.39x - 1.1$, $n = 121$), respectively.

Discussion

To overcome several disadvantages involved in conventional methods for PAP activity (1-8), we have developed a new assay and described its performance. DCAPP, a self-indicating synthetic substrate, has played a key role.

The K_m of DCAPP for PAP was 0.147 mmol/L in 0.1 mol/L citrate-HCl buffer (pH 5.4), close to that of DCNPP (0.137 mmol/L) already reported (8). However, the ϵ of the hydrolyzed product, DCAP, was 38% greater than that of DCNPP ($21\,490$ vs $15\,600 \text{ L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$) (8). Therefore, the DCAPP method is expected to be more precise than other methods even for low PAP activity. Moreover, our method is less susceptible to hemoglobin interference than the DCNPP method, and is applicable to automated analyzers because DCAP has a maximum absorption at 334.2 nm.

The pK_a of DCAP was shifted from pH 4.5 to 4.15 by adding albumin to the buffer solution. This increase in the ϵ of DCAP up to 15% in 0.1 mol/L citrate-HCl buffer (pH 5.4). The shift may be related to the acetyl radical ($C=O$ bond) forming resonance structures with amino groups of serum proteins in acidic conditions.

Because some serum albumin increases the rate of DCNPP hydrolysis (9), we added albumin to the assay buffer to give a final concentration of 3.8 g/L. We did not see any significant DCAPP hydrolysis, and obtained good correlations with the three methods except for the DCNPP method, which showed the bias already reported (9). The citrate buffer containing albumin was stable for 1 year at 4°C without any stabilizer.

The K_m value of PAP was 1.4×10^{-4} mol/L in 0.1 mol/L citrate-HCl buffer, whereas those of ACP from

blood cells (erythrocytes, leukocytes, & platelets) were $1.67\text{--}2.86 \times 10^{-3}$ mol/L when using DCAPP as the substrate. Since the K_m of PAP was 0.1 that of ACP from blood cells, the affinity of DCAPP for PAP turned out to be stronger than for ACP from blood cells (Table 1).

We selected 1.15×10^{-3} mol/L as the final substrate concentration at which the V_{max} was 89% with PAP. For PAP activity measurement, the substrate concentration should be $>1.15 \times 10^{-3}$ mol/L, but this will result in high blank value (>0.1 A), as shown in Fig. 1.

In this study, we measured PAP activity by L-tartrate inhibition. The PAP activity was inhibited by 98% with ≥ 20 mmol/L L-tartrate, whereas ACP from blood cells was inhibited by 0.6–20.1%. The inhibition rate of PAP in this study was in good agreement with that of the 1-NA method, whereas that of ACP from other organs was not (3). The inhibition of other organ enzymes ranged from 50.0% to 72.8%, but they were less than that of the enzyme from blood cells contaminated with serum. Consequently, the tartrate inhibitory method does not impair the specificity of the PAP activity measurement. The correlation between the present method and PAP EIA method ($r = 0.986$) validates the specificity.

In summary, for the measurement of PAP activity, our kinetic method involving the self-indicating substrate DCAPP showed satisfactory performance on automated analyzers. Moreover, the new method was free from albumin interference, unlike the DCNPP method.

References

1. Kind PRN, King EJ. Estimation of plasma phosphatase by determination of hydrolyzed phenol with aminoantipyrine. *J Clin Pathol* 1954;7:322–6.
2. Morin LG. Ammonium thymolphthalein monophosphate as a new substrate for alkaline and acid phosphatase determinations in serum. *Clin Chem* 1973;19:1135–8.
3. Roy AV, Brower ME, Hayden JE. Sodium thymolphthalein monophosphate: a new acid phosphatase substrate with greater specificity for the prostatic enzyme in serum. *Clin Chem* 1971;17:1093–102.
4. Gundlach G, Muhlhausen B. Untersuchungen zur Kupplung des 1-Naphthols mit Fast-Red-TR. *J Clin Chem Clin Biochem* 1980;18:603–10.
5. Hillmann G. Fortlaufend photometrische Messung der Sauer-Phosphatase-Aktivitat. *Z Klin Chem Klin Biochem* 1971;3:273–4.
6. Bais R, Edwards JB. An optimized continuous-monitoring procedure for semiautomated determination of serum acid phosphatase activity. *Clin Chem* 1976;22:2025–8.
7. Small CW, McNutt P. Interferences in the direct kinetic determination of acid phosphatase activity [Letter]. *Clin Chem* 1984;30:594–5.
8. Teshima S, Hayashi Y, Ando M. Determination of acid phosphatase in biological fluids using a new substrate 2,6-dichloro-4-nitrophenyl phosphate. *Clin Chim Acta* 1987;168:231–8.
9. Valcour AA, Bowers GN, McComb RB. Evaluation of a kinetic method for prostatic acid phosphatase with use of self-indicating substrate, 2,6-dichloro-4-nitrophenyl phosphate. *Clin Chem* 1989;35:939–45.
10. Kuroiwa K, Katayama K, Osawa S. Determination of acid phosphatase in serum using a new substrate, 2,6-dichloro-4-acetylphenyl phosphate [Abstract]. *Clin Chem* 1989;35:1117–8.
11. Foti AG, Herschman H, Cooper JF. Comparison of human prostatic acid phosphatase by measurement of enzymatic activity and by radioimmunoassay. *Clin Chem* 1977;23:95–9.
12. Miller JC, Miller JN. *Statistics for analytical chemistry*, 2nd ed. Chichester, UK: Ellis Horwood Ltd., 1989:57p.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s):	Engelhardt et al.)	
Serial No.:	08/479,997)	Group Art Unit: 1656
Filed:	June 7, 1995)	Ex'r: Alexander H. Spiegler
For:	OLIGO- OR POLYNUCLEOTIDES)	
	COMPRISING PHOSPHATE MOIETY)	
	LABELED NUCLEOTIDES)	
	(As Previously Amended))	

Assistant Commissioner for Patents
Washington, D.C. 20231

St. Louis, Missouri 63124

DECLARATION OF DR. CHARLES W. PARKER

I, Charles W. Parker, hereby declare as follows:

1. I am presently Professor *Emeritus* of Medicine, Department of Microbiology and Immunology at Washington University School of Medicine (WUSM), St. Louis, Missouri, having held that position since 1997. I am also Associate Physician at Barnes Hospital, also in St. Louis, Missouri. Prior to my present position at WUSM, I was Professor of Medicine there from 1971-1997. Overlapping with my position as Professor of Medicine, I was Professor of Microbiology, Immunology and Molecular Biology from 1975-1997. Within that same period of time (1977-1989), I was a full investigator of the Howard Hughes Medical Institute which funded me at Washington University for immunologic studies. From 1968 to 1971, I was Associate Professor of Medicine (WUSM). Before that, from 1963 to 1968, I was Assistant Professor of Medicine (WUSM). Earlier, from 1962 to 1988, I was the Head, Division of Allergy and Immunology (WUSM). From 1960 to 1963, I was

Instructor in Medicine (WUSM). My professional experience is listed on my *curriculum vitae* attached as Exhibit 1.

2. In terms of my education and research training, I entered Washington University as an undergraduate student in 1947. I entered Washington University School of Medicine (WUSM) in 1949, without having received my baccalaureate from Washington University. I received my medical degree (M.D.) from WUSM in 1953, having graduated *cum laude*. I was also a member of the Medical School's Chapter of the Alpha-Omega-Alpha Society (), which is a national academic honorary organization. After receiving my M.D. degree, I was an intern at Barnes Hospital from 1953 to 1954. I served in the United States Navy for two years (1954-1956) as a naval physician. After my naval discharge, I was later appointed as Assistant Resident at Barnes Hospital from 1956 to 1958, which was followed by my one year appointment as Chief Resident (1958-1959). I was in research training in immunology at WUSM from 1959 to 1962 and I remained at WUSM for the remainder of my professional career. From 1961 to 1962, I was a Research Fellow at the United States Public Health Service (USPHS). For a decade (1962-1972), I was supported by a research development award (Research Career Award) from the National Institutes of Allergy and Infectious Diseases (NIAID). A good deal of my research over the past five decades has involved conjugate chemistry and the use of conjugated products, including radiolabeled proteins, for immunization and radioimmunoassays. This research work done in collaboration with research fellows and faculty members at WUSM and elsewhere included original descriptions of radioimmunoassays for morphine and related drugs, the cardiac form of creatine phosphokinase for diagnosing heart attacks, cyclic nucleotides, prostaglandins, difficult to measure drugs, such as digitalis, and hepatitis antigens. A number of these assays are still used with little modification from our original methods. For example, screening for morphine (opiate addiction)

is still carried out with our immunization and immunoassay procedures. The cyclic nucleotide assays we developed had a profound effect on the field and are still used. Since its original description our assay for creatine phosphokinase has been a crucial diagnostic test for acute myocardial infarction and is still used today. We developed the creatine phosphokinase test in collaboration with cardiac researchers at WUSM. Our laboratory at WUSM is also well known for its work on drug allergies and lipid mediators of inflammation. My education and research experience are listed on my CV (Exhibit 1).

3. I am the author of over three hundred scientific publications which are listed on the last several pages in my CV (Exhibit 1). Included among those publications are six review articles on radioimmunoassays and the use of radiolabeled proteins in immunological studies.

4. As a student or researcher, I have received several honors and awards, including a Mosby Award, Phi Eta Sigma, Alpha Omega Alpha, Sigma Xi, a Hixon Award and a Bausch & Lomb Award. As indicated in the preceding paragraph, I received a Research Career Award from the NIAID (1962-1972). I received a Honorary Fellowship Award in 1983 from The American Academy of Allergy and Immunology for original research in the field of allergy and immunology. I also received a Washington University Alumni Award. WUSM has also honored me by establishing a scholarship in my name, The Charles W. Parker Medical Student Scholarship. My various honors and awards over the years are listed on my CV (Exhibit 1).

5. In terms of editorial responsibilities, I have been extensively involved over my professional and research career in reviewing and editing scientific manuscripts submitted for publication to leading research journals. Among such research

journals, I have been a member of the Editorial Board for the *Journal of Allergy and Clinical Immunology*, *Immunochemistry*, *Clinical Immunology and Immunopathology*, *Journal of Immunology* and the *Journal of Clinical Investigation*. From 1977-1982, I was Associate Editor for the *Journal of Clinical Investigation* and during that same period I was also Section Editor for the *Journal of Immunology* (1977-1982). I would consider the last two journals to be among the most prestigious and critically reviewed scientific journals in their fields. My extensive involvement in the review process affirms my conviction that responsible investigators must be willing to invest their own time in helping to maintain high research standards. My editorial responsibilities are listed on my CV (Exhibit 1).

6. Among the professional societies and organizations in which I have enjoyed membership and rank over the years are the following: the American Board of Internal Medicine, Central Society for Clinical Research, Fellow and Member of the American Academy of Allergy, American Association of Immunologists, Collegium Internationale Allergologicum, Association of American Physicians, American Heart Study Section (1972-1974), American Society for Clinical Investigation, Council of the American Society for Clinical Investigation (1973-1976) and the American Federation for Clinical Research. These various societies and organizations are listed on my CV (Exhibit 1).

7. Over the years I have had several consulting relationships and have served on several scientific boards. In particular, I spent approximately twenty years as a member of various review committees in the National Institutes of Health (NIH) which involved a minimum of three meetings per year. Since becoming Professor *emeritus* at WUSM, I have continued in this role as a reviewer for the National Center for Research Resources (NCRR). I have also been an adviser to a number of pharmaceutical companies, which included a position on the Board of Scientific

Advisers of the Roche Institute for Molecular Biology from 1978-1981. I have also been an *ad hoc* consultant to several leading pharmaceutical companies, including Searle, Merck, Pfizer, Eli Lilly, Mead Johnson, Abbott, Glaxo Wellcome and Nippon Zoki. My consulting relationships and board memberships are listed on my CV (Exhibit 1).

8. I have been asked by Enzo Life Sciences, Inc. (previously named Enzo Diagnostics, Inc.) to review as its scientific consultant significant portions of the prosecution history of United States Patent Application Serial No. 08/479,997, filed on June 7, 1995 in the name of Dean L. Engelhardt et al. as inventors. The title of the Engelhardt application is "Oligo- or Polynucleotides Comprising Phosphate Moiety Labeled Nucleotides." Included for my review were the following documents: the patent specification filed on June 7, 1995 (which I have been informed takes June 23, 1982 as its priority date); the former and previously pending claims (454-567) in this application; the November 26, 2001 Office Action; the December 27, 2001 Interview Summary, and various prior art documents cited in the aforementioned office action. The cited documents that I have reviewed include two scientific papers by Mark J. Halloran and Charles W. Parker ["The Preparation of Nucleotide-Protein Conjugates: Carbodiimides As Coupling Agents," Journal of Immunology 96:373-378 (1966); "The Production of Antibodies to Mononucleotides, Oligonucleotides and DNA," also Journal of Immunology 96:379-385)], and two U.S. patents [Ward et al., U.S. Patent No. 4,711,955; and Falkow et al., U.S. Patent No. 4,358,535]. I am also the same Charles W. Parker named on both aforementioned Halloran and Parker papers.¹ A

¹ For convenience, my first 1966 Journal of Immunology paper ["The Preparation of Nucleotide-Protein Conjugates: Carbodiimides As Coupling Agents," volume 96, pages 373-378] will hereinafter be referred to as Halloran I. My second 1966 paper ["The Production of Antibodies To Mononucleotides, Oligonucleotides and DNA," volume 96, pages 379-385] will be referred to as Halloran II.

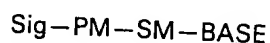
copy of each of Halloran I, Halloran II, the Ward patent and the Falkow patent are attached to my Declaration as Exhibits 2-5, respectively. I have also reviewed two declarations that were submitted in the Engelhardt application: Declaration of Dr. Cheryl H. Agris, Attorney At Law (In Support of the Written Description, Enablement & Non-Obviousness of the Invention Claimed in U.S. Patent Application Serial No. 08/479,997); and Declaration of Dr. Dean L. Engelhardt In Support of Adequate Description and Enablement. I believe that the Agris Declaration and the Engelhardt Declaration were originally submitted on January 18, 2001 and November 27, 1997, respectively. As part of my review, I have also read a set of claims 576-825² that will be submitted, together with my Declaration, in a paper to be filed in response to the November 26, 2001 Office Action. A copy of the aforementioned claims 576-825 to be submitted are attached to my Declaration as Exhibit 6.

9. Based upon my review of the claims (576-825) being submitted to the U.S. Patent Office (Exhibit 6), I believe that the invention in the Engelhardt application is directed to oligo- or polynucleotides comprising phosphate moiety labeled nucleotides. Such claimed oligo- or polynucleotides are useful as hybridization probes for detecting nucleic acids of interest. I believe that a third of the claimed embodiments in the Engelhardt application are directed to *non-polypeptide*, non-radioactive label moieties attached to the phosphate moiety of a modified nucleotide in an oligo- or polynucleotide (claims 576-657). The middle third of Engelhardt's claimed embodiments (claims 658-735) are directed to recited members for the non-radioactive label moiety Sig in such modified nucleotides in an

² I believe that new claims 576-735 correspond in large part to many of the former and previously pending claims 454-567. To the extent that similar if not identical subject matter is recited, the opinions and conclusions in my Declaration apply to the previously pending claims as well as the new claims. In addressing the art rejections in the November 26, 2001 Office Action below, I will be referring to various new claims but I will also list the corresponding former claims in footnotes to the rejections.

oligo- or polynucleotide. Such members can take the form of biotin, iminobiotin, an electron dense component, a magnetic component, a metal-containing component, a fluorescent component, a chemiluminescent component, a chromogenic component, a hapten and combinations of any of the foregoing. None of the following are included in the preceding list of Sig members: a polypeptide, a protein and an enzyme. The remaining third of Engelhardt's claimed embodiments (736-813) are directed to an oligo- or polynucleotide comprising at least one modified nucleotide in which a non-radioactive moiety label Sig is directly detected when indirectly attached to the phosphate moiety through a chemical linkage comprising a polypeptide or a protein. Finally, several dependent claims (814-825) define embodiments wherein Sig is covalently attached to PM or the phosphate through a chemical linkage comprising a polypeptide or a protein (claims 814, 817, 820 and 823). Other dependent claims define such polypeptide as comprising polylysine³ (claims 815, 818, 821 and 824) or such polypeptide or protein as being selected from avidin, streptavidin and anti-hapten immunoglobulin (claims 816, 819, 822 and 825).

A. Based upon my review, I believe that claims 576-595 describe one of the major compositions in the Engelhardt application. As set forth in claim 576, the claimed oligo- or polydeoxyribonucleotide, which is complementary to a nucleic acid of interest or a portion thereof, comprises at least one modified nucleotide having the formula



wherein PM is a phosphate moiety attached to SM, a sugar moiety, and BASE is a base moiety selected from the group consisting of a pyrimidine, a purine and a deazapurine, or analog thereof, the BASE being attached to SM. Sig is covalently attached to PM directly or through a chemical linkage, and Sig comprises a non-polypeptide, non-radioactive label moiety which can be directly or indirectly detected when attached to PM or when the modified nucleotide is incorporated into the oligo- or polydeoxyribonucleotide or when the oligo- or polydeoxynucleotide is hybridized to the complementary nucleic acid of interest or a portion thereof. I believe that claims 577-595 depend from claim 576 and are directed to various other specific embodiments, such as the nature of Sig

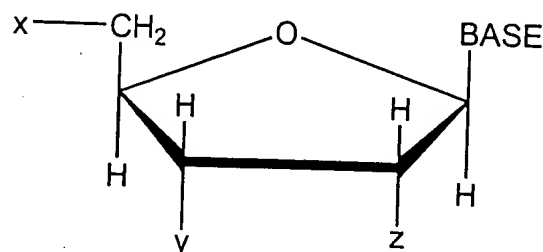
³ Polylysine belongs to the class of compounds called polyamino acids.

(claims 577-578 and 586-591); the covalent attachment of Sig (claims 579 and 592); the chemical linkage (claims 580-584); the nature of PM (claim 585); the nature of SM (claim 593-594); and the inclusion of at least one ribonucleotide (claim 595).

(i) I believe that claims 658-676 differ from claims 576-595 in two respects. First, independent claim 658 does not recite the term "non-polypeptide" for the non-radioactive label moiety Sig. Second, claim 658 recites specific members for Sig (biotin, iminobiotin, an electron dense component, a magnetic component, a metal-containing component, a fluorescent component, a chemiluminescent component, a chromogenic component, a hapten and a combination). The claims that depend from claim 658 are also directed to specific embodiments, such as nature of Sig (claims 659-660 and 668-672); the covalent attachment of Sig (claims 661 and 673); the chemical linkage (claims 662-666); the nature of PM (claims 667); the nature of SM (claims 674-675); and the inclusion of at least one ribonucleotide (claim 676).

(ii) I also believe that claims 736-754 differ from the above-described claims 576-595 and 658-676 as follows. Claim 736 is independent and it recites that Sig is covalently attached to PM through a chemical linkage comprising a polypeptide or a protein. Thus, for the non-radioactive label moiety Sig, claim 736 recites neither the term "non-polypeptide" nor the Sig members. Claims 737-754 are directed to further more specific embodiments of claim 736. These dependent claims define the nature of Sig (claims 737-738 and 742-750); the covalent attachment of Sig (claims 739 and 751); the chemical linkage (claims 740 and 748-750); the nature of PM (claim 741); the nature of SM (claims 752-753); and the inclusion of at least one ribonucleotide (claim 754).

B. From my review I believe that claims 596-616 define another aspect of the Engelhardt invention. As set forth in claim 596, the invention claimed in the Engelhardt Declaration is also directed to an oligo- or polydeoxyribonucleotide which is complementary to a nucleic acid of interest or a portion thereof. Such oligo- or polydeoxyribonucleotide comprises at least one modified nucleotide having the structural formula:



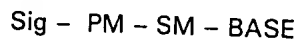
wherein BASE is a moiety selected from the group consisting of a pyrimidine, a purine and a

deazapurine, or analog thereof, the BASE being attached to the 1' position of the pentose ring from the N1 position when BASE is a pyrimidine or from the N9 position when BASE is a purine or a deazapurine. The elements x, y and z are each selected from the group consisting of H—, HO—, a mono-phosphate, a di-phosphate and a tri-phosphate. Sig is defined in claim 596 as being covalently attached directly or through a chemical linkage to at least one phosphate selected from the group consisting of x, y, z, and a combination thereof. Furthermore, Sig comprises a non-polypeptide, non-radioactive label moiety which can be directly or indirectly detected when so attached to the phosphate or when the modified nucleotide is incorporated into the oligo- or polydeoxynucleotide or when the oligo- or polydeoxynucleotide is hybridized to the complementary nucleic acid of interest or a portion thereof. Claims 597-616 depend from claim 596 and they define various narrower embodiments, including the nature of Sig (claims 597-598 and 606-611); covalent attachment of Sig (claims 599 and 612); the chemical linkage (claims 599-604); the nature of x, y and/or z (claim 605 and 613-614); the inclusion of at least one ribonucleotide (claim 615); and a structural formula for the claimed oligo- or polydeoxyribonucleotide (claim 616).

(i) I believe that claims 677-696 differ from claims 596-616 in the following respects. First, independent claim 677 does not recite "non-polypeptide" for the non-radioactive label moiety Sig. Second, specific members for Sig are recited in claim 677 (biotin, iminobiotin, an electron dense component, a magnetic component, a metal-containing component, a fluorescent component, a chemiluminescent component, a chromogenic component, a hapten and a combination). Dependent claims 678-696 are directed to various embodiments, such as the nature of Sig (claims 678-679 and 687-691); the covalent attachment of Sig (claims 680 and 692); the chemical linkage (claims 681-685); the elements x, y and/or z (claims 686 and 693-694); the inclusion of at least one ribonucleotide (claim 695); and the structural formula of the oligo- or polydeoxyribonucleotide (claim 696).

(ii) I also believe that claims 755-774 differ from claims 596-616 and 677-696 described above. First, claim 755, an independent claim, recites that Sig is covalently attached to PM through a chemical linkage comprising a polypeptide or a protein. Second, for the non-radioactive label moiety Sig, claim 755 lacks the recitation of the term "non-polypeptide" and the various members of Sig. Dependent embodiments are provided in claims 756-774 and include the nature of Sig (claims 756-757 and 761-766); the covalent attachment of Sig (claims 758 and 770); the chemical linkage (claims 759 and 767-769); the elements x, y and/or z (claims 760 and 771-772); the inclusion of at least one ribonucleotide (claim 773); and a structural formula for the claimed oligo- or polydeoxyribonucleotide (claim 774).

C. I believe that another aspect of the Engelhardt invention is defined in claims 617-636. In claim 617, the oligo- or polynucleotide is also complementary to a nucleic acid of interest or a portion thereof, and it comprises at least one modified nucleotide having the formula



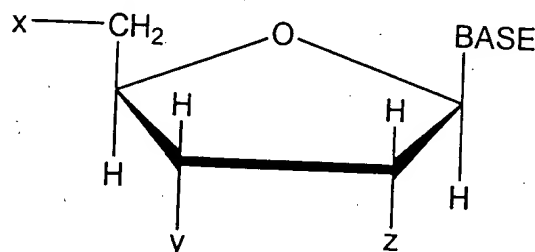
wherein PM is a phosphate moiety, SM is a sugar moiety and BASE is a moiety selected from the group consisting of a pyrimidine, a purine and a deazapurine, or analog thereof. PM is attached to SM, BASE is attached to SM, and Sig is covalently attached to PM directly or via a chemical linkage. Sig comprises a non-polypeptide, non-radioactive label moiety which can be directly or indirectly detected when attached to PM or when the modified nucleotide is incorporated into the oligo- or polynucleotide, or when the oligo- or polynucleotide is hybridized to the complementary nucleic acid of interest or a portion thereof, provided that when the oligo- or polynucleotide is an oligoribonucleotide or a polyribonucleotide, and when Sig is attached through a chemical linkage to a terminal PM at the 3' position of a terminal ribonucleotide, the chemical linkage is not obtained through a 2',3' vicinal oxidation of a 3' terminal ribonucleotide previously attached to the oligoribonucleotide or polyribonucleotide. Claims 618-636 are dependent embodiments and they include: the nature of Sig (claims 618-619 and 627-632); the covalent attachment of Sig (claims 620 and 633); the chemical linkage (claims 621-625); the nature of PM (claim 626); the nature of SM (claims 634-635); and the inclusion of at least one deoxyribonucleotide (claim 636).

(i) I believe that claims 697-715 are different from claims 617-636 in two respects. First, independent claim 697 does not recite the term "non-polypeptide" for the non-radioactive label moiety Sig. Second, claim 697 lists specific Sig members (biotin, iminobiotin, an electron dense component, a magnetic component, a metal-containing component, a fluorescent component, a chemiluminescent component, a chromogenic component, a hapten and a combination). Other various aspects are given in claims 698-715 including the nature of Sig (claims 698-699 and 707-711); the covalent attachment of Sig (claims 700 and 712); the chemical linkage (claims 701-705); the nature of PM (claim 706); the nature of the sugar moiety (claims 713-714); and the inclusion of at least one deoxyribonucleotide (claim 715).

(ii) My review also shows that claims 775-793 differ from the afore-described claims 617-636 and 697-715 as follows. Unlike its counterparts (claims 617 and 697), independent claim 775 recites that Sig is covalently attached to PM via a chemical linkage comprising a polypeptide or a protein. Moreover, claim 775 does not recite the term "non-polypeptide" or the members of the non-radioactive label moiety Sig, unlike claims 617 and 697.

Dependent embodiments of claim 775 are given in claims 776-793. These embodiments include the nature of Sig (claims 776-777 and 781-785); the covalent attachment of Sig (claims 778 and 790); the chemical linkage (claims 779 and 787-789); the nature of PM (claim 780); the nature of SM (claims 791-792); and the inclusion of at least one deoxyribonucleotide (claim 793).

D. My review also shows me that another composition claimed in the Engelhardt application is an oligo- or polynucleotide as set forth in claims 637-657. As given by claim 637, this claimed composition is complementary to a nucleic acid of interest or a portion thereof, such oligo- or polynucleotide comprising at least one modified nucleotide having the structural formula:



BASE is a moiety selected from the group consisting of a pyrimidine, a purine and a deazapurine, or analog thereof, and it is attached to the 1' position of the pentose ring from the N1 position when BASE is a pyrimidine or from the N9 position when BASE is a purine or a deazapurine. The elements x, y and z are each selected from the group consisting of H—, HO—, a mono-phosphate, a di-phosphate and a tri-phosphate. Sig is covalently attached directly or through a chemical linkage to at least one phosphate selected from the group consisting of x, y and z, and a combination thereof. Sig also comprises a non-polypeptide, non-radioactive label moiety which can be directly or indirectly detected when so attached to the phosphate or when the modified nucleotide is incorporated into the oligo- or polynucleotide, or when the oligo- or polynucleotide is hybridized to the complementary nucleic acid of interest or a portion thereof. It is provided in the language of claim 637 that when the oligo- or polynucleotide is an oligoribonucleotide or a polyribonucleotide and when Sig is attached through a chemical linkage to a terminal PM at the 3' position of a terminal ribonucleotide, the chemical linkage is not obtained through a 2',3' vicinal oxidation of a 3' terminal ribonucleotide previously attached to the oligoribonucleotide or polyribonucleotide. Claims 638-657 are dependent embodiments directed to the nature of Sig (claims 638-639 and 647-652); the covalent attachment of Sig (claims 640 and 653); the chemical linkage (claims 641-645); the nature of elements x, y and/or z (claims 646 and 654-655); the inclusion of at least one deoxyribonucleotide (claim 656); and the structural formula for the oligo- or polynucleotide (claim 657).

(i) Based on my review, I also believe that claims 716-735 differ from claims 637-657 as follows. Claim 716, an independent claim, does not recite the term "non-polypeptide" for the non-radioactive label moiety Sig. Further, specific Sig members are listed in claim 716 (biotin, iminobiotin, an electron dense component, a magnetic component, a metal-containing component, a fluorescent component, a chemiluminescent component, a chromogenic component, a hapten and a combination). Other embodiments are given in claims 717-735, including the nature of Sig (claims 717-718 and 726-730); the covalent attachment of Sig (claims 719 and 731); the chemical linkage (claims 720-724); the nature of x, y and/or z (claims 725 and 732-733); the inclusion of at least one deoxyribonucleotide (claim 734); and the structural formula of the oligo- or polynucleotide (claim 735).

(ii) Claims 794-813 are different from claims 637-657 and 716-735 described above. Independent claim 794 recites that Sig is covalently attached to PM via a chemical linkage comprising a polypeptide or a protein. Unlike claims 637 and 716, claim 794 lacks the recitation for "non-polypeptide" and the various members for the non-radioactive label moiety Sig. Claims 795-813 depend from claim 794 and provide other embodiments including the nature of Sig (claims 794-795 and 800-805); the covalent attachment of Sig (claims 797 and 809); the chemical linkage (claims 798 and 806-808); the nature of PM (claim 799); the elements x, y and/or z (claims 799 and 810-811); the inclusion of at least one deoxyribonucleotide (claim 812); and a structural formula for the claimed oligo- or polynucleotide (claim 813).

E. As I indicated in Paragraph 9 above, several dependent claims (814-825) define embodiments wherein the non-radioactive label moiety Sig is attached indirectly to the phosphate moiety through a polypeptide or protein chemical linkage. Thus, such dependent claims 814, 817, 820 and 823 recite that the non-radioactive label moiety Sig is covalently attached to PM (or to at least one phosphate) through a chemical linkage comprising a polypeptide or a protein. In turn, claims 815, 818, 821 and 824 define the polypeptide as comprising polylysine. Other dependent claims (816, 819, 822 and 825) define such polypeptide or protein as being selected from avidin, streptavidin and anti-hapten immunoglobulin.

10. I believe that in the November 26, 2001 Office Action, ten so-called "art" rejections (Paragraphs 7-9 and 11-17 in the Office Action) were raised against the former claims 454-567. Eight of the ten rejections (Rejections Nos. 2 through 9,

Dean L. Engelhardt et al.

Serial No. 08/479,997

Filed: June 7, 1995

Page 13 [Declaration of Dr. Charles W. Parker]

Paragraphs 8-9 and 11-16 in the Office Action) concerned either one or the other of my 1966 Journal of Immunology papers (Halloran I or Halloran II). My remarks in Paragraphs 15(A) through (H) below are directed to Rejections Nos. 2 through 9 (Paragraphs 8-9 and 11-16) in the November 26, 2001 Office Action.

11. As Enzo's scientific consultant, I am making this Declaration in support of the novelty and non-obviousness of the subject matter claimed in the Engelhardt application. I am also being compensated by Enzo for making this Declaration on its behalf.

12. Based upon my own training, background and experience, I would respectfully submit that at the time this application was first filed in June 1982, a person of ordinary skill in the art relevant to the subject matter being claimed, including nucleic acid chemistry and modification, which would include the attachment of labels and linker arms to nucleotides and nucleic acids, and nucleic acid detection and detection formats, would have possessed or could have been actively pursuing an advanced degree in organic chemistry and/or biochemistry. A person of ordinary skill in the art might also possess some knowledge about protein chemistry, including protein modification, labeling and detection, although such knowledge would not approach his or her knowledge about nucleic acid chemistry, nucleic acid modification and labeling, and nucleic acid detection and formatting. Such an ordinarily skilled person could also be at least approaching or ranging toward the level of a junior faculty member with 2-5 years of relevant experience, or would at least be a postdoctoral student with several years of experience. I consider myself to possess the level of skill and knowledge of at least a person of ordinary skill in the art to which the present application and invention pertains.

13. As a person of ordinary skill in the art, it is my opinion and conclusion that the invention in the Engelhardt application as set forth in claims 576-825 being submitted to the Patent Office is novel over my two 1966 papers (Halloran I or Halloran II). Further, it is my opinion and conclusion that the invention claimed in the Engelhardt application would not have been rendered obvious over either of my two 1966 papers (Halloran I or Halloran II), either by themselves, or in combination with each other or with either or both of the Ward and Falkow U.S. patents cited in the November 26, 2001 Office Action. My reasons are set forth in the following paragraphs.⁴

14. Before discussing my reasons, I would like to provide some background to my 1966 papers (Halloran I and Halloran II).

A. Our two 1966 papers (Halloran I and Halloran II) were published in the Journal of Immunology, the leading publication of the American Association of Immunologists. Both papers concerned conjugated products for use in immunology. Halloran I and Halloran II were published in a journal devoted to immunology, which was not at that time, normally read by molecular biologists or investigators in the field of molecular biology. In noting the earlier works of H.G. Khorana who had used *water-insoluble* carbodiimides for his work in synthesizing oligonucleotides and Gertrude E. Perlmann at Rockefeller in the 1950s who had studied phosphorus linkages in phosphoproteins, casein and pepsin, we prepared conjugates of protein covalently linked to mononucleotides, oligonucleotides and DNA using *water-soluble* carbodiimides, such as ECDI and CMC. Water-soluble carbodiimides readily couple mono- and oligonucleotides to proteins in aqueous solution. Because we were initially not entirely sure which amino acid residues of proteins were involved in the coupling, we compared polylysine with other

⁴ Any opinions and conclusions given in this Declaration are done in light of my training, background

Dean L. Engelhardt et al.

Serial No. 08/479,997

Filed: June 7, 1995

Page 15 [Declaration of Dr. Charles W. Parker]

polymers containing different functional groups. We found that N-P linkages were readily formed with polylysine, were sufficiently stable for our purposes and were likely to be the predominant linkage in proteins. I should point out that two publications cited as References 5 and 20 in Halloran I disclosed non-aqueous conjugations and virtually non-aqueous conjugations, respectively, and as such, probably would not be suitable for reactions involving unblocked oligonucleotides. Copies of each of H. G. Khorana's 1961 paper [Khorana, H. G. and Vizsolyi, J. P., "Studies on Polynucleotides. VIII. Experiments on the Polymerization of Mononucleotides. Improved Preparation and Separation of Linear Thymidine Polynucleotides. Synthesis of Corresponding Members Terminated in Deoxycytidine Residues," Journal of American Chemical Society 83:675-685], and Michael Sela's 1964 paper ["Sela et al., "Uridine-Specific Antibodies Obtained With Synthetic Antigens," Proceedings National Academy of Science 52:285-292] are attached to my Declaration as Exhibits 7 and 8, respectively. In Sela's 1964 paper, the phosphate group of a mononucleotide was converted to a COOH group for coupling, but oligonucleotide coupling was not studied. In addition to polylysine, we also conjugated oligonucleotides and DNA to other polypeptides, including human serum albumin (HSA) and bovine γ -globulin (B γ G). We also modified polyamino acids chemically to put aliphatic hydroxy groups on them. In the case of polylysine, we modified it with ethylene oxide to prepare hydroxylated polylysine.⁵ Our purpose in preparing nucleotide-protein conjugates was primarily to use such conjugates to induce antibody formation with nucleotide specificity,

and experience as a person of at least ordinary skill in the art.

⁵ It should also be noted that our conjugation method was adaptable to polyamino acids containing other amino acids together with lysine (lysine and other amino acids are copolymerized; for example, poly-D,L-alanyl-L-lysyl mixed copolymer). In addition to biotin, as described in Example V (page 57) in the Engelhardt specification, oligonucleotides conjugated polypeptides in which a portion of the lysyl ϵ -ammonium groups are still free so that they can be readily reacted with a variety of amino agents (for example, succinic anhydride, luminol imidoesters and 2,4-dinitrophenol sulfonic acids (if desired). Thus, a wide variety of systematic modifications are possible.

and secondarily to study DNA and RNA structure. In the very first paragraph in Halloran I, we disclosed:

For some time it has been apparent that antibodies might be useful in the study of fine structure. A major stumbling block has been the unavailability of a method which would render polynucleotides antigenic yet largely preserve their structural integrity. In approaching the problem of covalently conjugating polynucleotides to proteins as a means of stimulating antibody formation, we sought a procedure which would employ terminal nucleotide PO₄ or OH groups for coupling.

...
... Evidence will be presented in this communication which indicates that nucleotides do couple to proteins and polylysine under very mild conditions, in the presence of carbodiimides. Investigation strongly implicates formation of N-P bonds as the principle type of linkage. In the accompanying article [Halloran II] it will be shown that conjugates of proteins with mononucleotides, oligonucleotides and DNA elicit the formation of antibodies with nucleotide specificity (2). A brief resume of this work has been reported earlier (3).

Halloran I, Page 373, Left Column

The formation of N-P-O bonds is depicted in Figure 1 of Halloran I (see page 374, Exhibit 2).

B. In preparing the nucleotide-protein conjugates described in Halloran I and used for antibody formation in Halloran II, we were quite concerned with minimizing the modifications or changes to the nucleic acid polymer or monomer being coupled to the protein. In Halloran I, page 378 (left column), we disclosed:

In the method of conjugation described in this article, mononucleotides, oligonucleotides, and DNA are coupled directly to protein. By contrast, the methods described in the preceding paragraph require some form of preliminary chemical modification of the mononucleotide, nucleoside or base. The applicability of these methods to coupling large units (oligo- and polynucleotides) to proteins or polyamino acids has not been established. We believe, therefore, that the general method described herein has certain advantages over previously described procedures.

In essence, we were preparing nucleotide-protein conjugates in which the nucleotide or polynucleotide portion of the conjugate was directly coupled to the protein without an additional chemical linkage or linker arm and with minimal changes to the nucleotide or polynucleotide.

C. Other investigations of the antigenicity of nucleic acids using conjugates followed our 1966 papers. Another paper by Michael Sela's group published in 1970 [Bonavida et al., "Antibodies To Transfer RNA Obtained With Covalently Linked tRNA Conjugates," Biochemical and Biophysical Research Communications 41:1335-1341] dealt with obtaining or precipitating anti-RNA antibodies to tRNA-BSA conjugates which were analyzed with tRNA radioactively labeled with ³²P. Transfer RNAs (tRNAs) are polymers of ribonucleotides with approximately 75 nucleotide residues. A copy of Sela's 1970 paper ["Antibodies to Transfer RNA Obtained with Covalently Linked tRNA Conjugates," Biochemical and Biophysical Research Communications 41:1335-1341 (1970)] is attached to my Declaration as Exhibit 9. Sela (1970) cited our first 1966 paper (Halloran I) for using ECDI "for the coupling of mononucleotides to BSA" (bovine serum albumin) [although we had also demonstrated coupling to oligonucleotides and polynucleotides].

D. To my knowledge and belief, the fields of nucleic acid technology and molecular biology did not seize upon our two 1966 immunological investigations (Halloran I and Halloran II) involving protein-polynucleotide conjugates and their use in producing antibodies for studying nucleic acid structure. I note that a considerable period of time, sixteen years in fact, passed between our two 1966 publications and the filing of Enzo's original patent application in June 1982. It is my opinion and conclusion that the Engelhardt application appears to be the first

real and practical application of our methodology for making protein-polynucleotide conjugates to the field of non-radioactive labeled nucleic acid probe technology. Because our own investigations were aimed at producing antibody formation with nucleotide specificity, we were not motivated or inclined to include any label, let alone a non-radioactive label in our nucleotide-protein conjugates (although we did employ crude non-specific protein and DNA stains, as discussed below). Thus, neither my Halloran I nor Halloran II papers disclosed or showed the use of a non-polypeptide non-radioactive label moiety Sig, as required by a number of claims in the Engelhardt application. Although protein and DNA stains in the form of Amidoschwartz and Feulgen⁶ were disclosed in Halloran I, these are non-specific stains that do not generate a sensitive, hybridization signal, as contrasted to Engelhardt's claimed oligo- or polynucleotides in which the non-polypeptide, non-radioactive label moiety Sig (such as the biotin moiety disclosed throughout the Engelhardt specification and used in Example V is covalently attached to a modified nucleotide. Feulgen and Amidoschwartz stains were used in our investigation for the sole purpose of showing that protein and oligonucleotide co-migrated during electrophoresis in a dissociating gel. Clearly, our nucleotide-protein conjugates did not include a modified nucleotide comprising a non-radioactive label moiety Sig, as set forth in various Engelhardt claims. Moreover, we never thought of attaching such a non-radioactive label moiety Sig to the phosphate moiety through a chemical linkage comprising a polypeptide or a protein, as set forth in other Engelhardt claims. As a person of at least ordinary skill in the art, I wish to make it clear that I did not intend nor was I motivated to use the conjugated poly-L-lysine (or other polypeptides, such as HSA and ByG) as a chemical linkage to which a non-radioactive label moiety (such as Engelhardt's claimed Sig) could be attached indirectly to the phosphate moiety of a modified nucleotide in an oligo- or polynucleotide. At the time that I published my two 1966 papers, I would not

⁶ Amidoschwartz and Feulgen compounds are used to stain protein and DNA, respectively.

have been motivated or even inclined to use the non-radioactive labels now being claimed in the Engelhardt application. Even if I had been motivated to include a detectable label (and I was not motivated to do so), I would have likely chosen to use radioactive isotopes, such as ^{32}P or radioactive iodine. Since the γ ^{32}P ATP label for ^{32}P end labeling of oligonucleotides was readily prepared or available and has a high specific activity, I most likely would have chosen radioactive ^{32}P for labeling in my investigations.

15. Having provided some background to my 1966 papers, I now wish to address each of the eight rejections (Nos. 2 through 9) set forth in the November 26, 2001 Office Action.

(A) The Second Rejection (Anticipation/Obviousness)

(i) With respect to new claims 576, 595-596 and 615-616,⁷ it is my opinion and conclusion as a person of ordinary skill in the art that the subject matter of these claims now being submitted to the Patent Office (attached as Exhibit 6) are novel over my cited publication (Halloran I). First, the Sig non-radioactive label moiety in any of the Engelhardt claims is not a polypeptide, which would include proteins or polyamino acids with lysyl residues such as disclosed in Halloran I. This applies to new claims 576-587, 588-589, 590-591, 592-607, 608-609, 610-611, 612-630, 631-632, 633-648, 649-650, 651-652 and 653-657.⁸ Second, none of the other new Engelhardt claims (658-735) recite a polypeptide, or protein, including poly-L-lysine, in the Markush members for Sig now listed in the new claims. If anything, the claims in the Engelhardt application

⁷ New claims 576, 595-596 and 615-616 correspond to former claims 454, 481-482 and 509-510.

⁸ New claims 576-587, 588-589, 590-591, 592-607, 608-609, 610-611, 612-628, 629-630, 631-632, 633-648, 649-650, 651-652 and 653-657 correspond to former claims 454-465, 467-468, 470-471, 478-493, 495-496, 498-499, 506-522, 524-525, 527-528, 535-550, 552-553, 555-556 and 563-567.

eschew any connection to the poly-L-lysine disclosed in my 1966 papers. Finally, with respect to other new Engelhardt claims (736-813), a modified nucleotide is cited in which a non-radioactive label moiety Sig is attached indirectly to the phosphate moiety through a chemical linkage comprising a polypeptide or a protein. Neither of my 1966 papers, including Halloran I, disclosed or even suggested that the poly-L-lysine or any protein could or should be used as a chemical linkage to attach a non-radioactive label moiety to the phosphate moiety, as set forth in such Engelhardt claims.

(ii) With respect to new claims 577 and 597,⁹ it is my opinion and conclusion that by excluding and not reciting polypeptides (or proteins or polyamino acids including poly-L-lysine), as non-radioactive label moieties, both claims are novel over Halloran I. The very fact that Halloran I discloses Amidoshwartz and Feulgen compounds for staining protein and DNA, respectively, does not reach the subject matter of the Engelhardt claims in which the non-radioactive label moiety Sig is either a non-polypeptide, does not have a polypeptide or a protein among the members of Sig, or only uses a polypeptide or protein as a chemical linkage to attach Sig to a phosphate moiety. It is my opinion and conclusion that the use of a stain, such as Amidoshwartz or Feulgen, has no connection either in structure or function to the non-polypeptide, non-radioactive label moiety Sig in the Engelhardt claims. In the case of Amidoshwartz and Feulgen compounds, these are crude stains that are not quantitative at all. These stain non-specifically to any proteins or DNA, without regard to such proteins or DNA being modified. Furthermore, due to either relative insensitivity, conditions of use, and inability to be amplified, Amidoshwartz and Feulgen stains do not generate a useful non-radioactive hybridization signal from an oligo- or polynucleotide comprising at least one *modified* nucleotide that comprises the non-radioactive label moiety Sig, as set forth in the Engelhardt claims. In the case of my cited paper, Halloran I, the

Amidoschwartz and Feulgen stains were simply used to show that the nucleotide or polynucleotide co-migrated with the protein in the dissociating gel in regions distinct from regions where the unmodified proteins and oligonucleotides migrated. To explain it in another way, there was a unique complex staining with both stains in contrast to other regions where neither of the precursors stained, implying, therefore, that the protein and nucleotide or polynucleotide were bound together in the gel. In short, such stains, such as the Amidoschwartz and Feulgen stains were not suitable as hybridization signals, and as far as I know have not been used for that purpose.¹⁰ As a person of ordinary skill in the art, I would not have used nor would I have contemplated using Amidoschwartz or Feulgen stains to detect a hybridization signal because their crude and non-specific properties make such use unsuitable.

(iii) With respect to new claims 578, 580, 586, 592, 593, 598, 600, 606 and 612-614,¹¹ the fact that Halloran I discloses the addition of proteins or polypeptides (such as HSA [human serum albumin] and poly-lysine) which comprise at least three carbon atoms is irrelevant to the Engelhardt claims, which specifically eschew polypeptides, do not recite polypeptide among members of the non-radioactive label moiety Sig, or recites that Sig is attached to the phosphate moiety through a chemical linkage comprising a polypeptide or a protein.

(iv) With respect to new claims 579, 581, 585, 594, 599, 601, 605 and 614,¹² again, the fact that Halloran I discloses the covalent attachment of -P-O-, the chemical linkage of CH₂-NH-, and the covalent attachment of Sig to PM

⁹ New claims 577 and 597 correspond to former claims 455 and 483.

¹⁰ Another reason discouraging the use of the Feulgen reaction for generating a signal from hybridized nucleic acid has to do with the harsh acid conditions under which the staining procedure is carried out. Under acidic conditions (for example, 1.0 N hydrochloric acid at 60°C for 5-20 minutes), the nucleic acid sought to be detected in a hybridized duplex may well be broken down or eluted altogether, with concomitant deterioration of signal.

¹¹ New claims 578, 580, 586, 592, 593, 598, 600, 606 and 612-614 correspond to former claims 456, 458, 464, 478, 479, 484, 486, 492 and 506-508.

through a phosphorus atom or phosphate oxygen, is irrelevant to the Engelhardt claims which specifically eschew polypeptide, or that do not recite polypeptide among members of the non-radioactive label moiety Sig, or recite that Sig is attached to the phosphate moiety through a polypeptide or protein chemical linkage.

(v) With respect to the Examiner's comments in the Office Action that even though Halloran does not teach the hybridization of an oligodeoxynucleotide to a nucleic acid of interest, or a portion thereof, it is an inherent property of an oligonucleotide to hybridize to a nucleic acid of interest (or portion thereof), which is complementary to the oligonucleotide, it is my opinion and conclusion that the Engelhardt claims are novel over Halloran I for reasons stated above. The Engelhardt claims specifically eschew the notion of a polypeptide as a non-radioactive detectable Sig moiety, or the claims do not recite a polypeptide among the members of Sig, or they include a polypeptide or a protein only as a chemical linkage for attaching Sig to the phosphate moiety.

(vi) It is my opinion and conclusion that it would not have been obvious to one of ordinary skill in the art at the time the invention was made to have used the oligonucleotide preparation method of Halloran I for conjugating a protein to an oligonucleotide (through the PM), in order to have produced the oligo- or polynucleotide compositions claimed in the Engelhardt application. As stated earlier, the Engelhardt claims either specifically eschew any notion of polypeptide or they do not include polypeptide among members of the detectable non-radioactive label moiety Sig, or the claims only include polypeptide or protein as a chemical linkage for attaching Sig to the phosphate moiety.

(B) The Third Rejection (Anticipation/Obviousness)

¹² New claims 579, 581, 585, 594, 599, 601, 605 and 614 correspond to former claims 457, 459, 463, 480, 485, 487, 491 and 508.

(i) With respect to new claims 617, 636, 637 and 656¹³, it is my opinion and conclusion that Halloran II does not disclose the subject matter of these claims. These and other claims in the Engelhardt application are directed to compositions in which Sig comprises a non-polypeptide, non-radioactive detectable label moiety, or to other compositions in which polypeptides or proteins are not listed among members of Sig, or in which a polypeptide or a chemical linkage is only used for attaching Sig to the phosphate moiety.

(ii) With respect to new claims 618 and 638,¹⁴ it is my opinion and conclusion that by excluding and not reciting polypeptides (or proteins or poly-L-lysine) as non-radioactive label moieties, both claims are novel over Halloran II. Thus, the fact that Halloran II discloses the use of Amidoschwartz and Feulgen staining to stain the protein and DNA, respectively, does not reach the subject matter of the Engelhardt claims in which the non-radioactive label moiety Sig is neither a polypeptide, nor is recited among Sig members as a polypeptide or protein. Moreover, the use of a stain, such as Amidoschwartz or Feulgen, has no connection to the non-polypeptide, non-radioactive label moiety Sig in the Engelhardt claims. In the case of the stains, Amidoschwartz and Feulgen are crude procedures which are not quantitative. Furthermore, it is my opinion and conclusion that these stains do not generate a useable non-radioactive signal from an oligo- or polynucleotide comprising a modified nucleotide that comprises the non-radioactive label moiety Sig, as set forth in the Engelhardt claims and invention.

(iii) With respect to new claims 619, 621, 627, 633-634, 639, 641, 647, 653 and 654,¹⁵ it is my opinion and conclusion that the fact that Halloran II discloses the addition of proteins or polypeptides (such as HSA [human serum

¹³ New claims 617, 636, 637 and 656 correspond to former claims 511, 538, 539 and 567.

¹⁴ New claims 618 and 638 correspond to former claims 512 and 540.

¹⁵ New claims 619, 621, 627, 633-634, 639, 641, 647, 653 and 654 correspond to former claims 513, 515, 521, 535-536, 541, 543, 549, 563 and 564.

albumin] and polylysine) which comprise at least three carbon atoms is irrelevant to the Engelhardt claims now being submitted. Such claims either specifically eschew any connection to polypeptide, or they do not recite polypeptide among members of the non-radioactive label moiety Sig, or they include a polypeptide or a protein only as a chemical linkage for attaching Sig to the phosphate moiety.

(iv) With respect to new claims 620, 622, 626, 635, 640, 642, 646 and 655,¹⁶ it is my opinion and conclusion that the fact that Halloran II discloses the covalent attachment of $-P-O-$, the chemical linkage of CH_2-NH- , and the covalent attachment of Sig to PM through a phosphorus atom or phosphate oxygen, is also irrelevant to the Engelhardt claims. The Engelhardt claims specifically eschew any connection to polypeptide, or they do not recite polypeptide (or protein or enzyme) among members of the non-radioactive label moiety Sig, or they recite a polypeptide or a protein only as a chemical linkage for attaching Sig to the phosphate moiety.

(v) With respect to the Examiner's comments in the Office Action that even though Halloran II does not teach the hybridization of an oligoribonucleotide to a nucleic acid of interest, or a portion thereof, it is an inherent property of an oligoribonucleotide to hybridize to a nucleic acid of interest (or portion thereof), which is complementary to the oligonucleotide, it is my opinion and conclusion that the Engelhardt claims are novel over Halloran I for reasons stated earlier. The Engelhardt claims either specifically eschew any connection to a polypeptide as a non-radioactive detectable Sig moiety, or the claims do not recite a polypeptide among the members of Sig, or they include a polypeptide or a protein only as a chemical linkage to attach Sig to the phosphate moiety.

(vi) It is my opinion and conclusion that it would not have been obvious to one of ordinary skill in the art at the time the invention was made to have modified

¹⁶ New claims 620, 622, 626, 635, 640, 642, 646 and 655 correspond to former claims 514, 516, 520, 537, 542, 544, 548 and 565.

the method of Halloran II (pages 373-378) so as to have conjugated a protein to an RNA or DNA molecule in order to have achieved an equally effective compound for use in hybridization, and thereby reach the claimed Engelhardt compositions. It is also my opinion and conclusion that if it had been that obvious, someone would have done so in the sixteen years following the publication of Halloran II and the filing of the Engelhardt application. In fact, I believe that no one did this before Engelhardt et al. filed their application in June 1982. As indicated earlier, all of the compositions in the Engelhardt application are directed to a non-polypeptide, non-radioactive label moiety Sig, or to members of Sig that do not include a polypeptide or protein, or to a polypeptide or protein chemical linkage to attach Sig to the phosphate moiety. This stands in contrast to my cited paper, Halloran II.

(C) The Fourth Rejection (Obviousness)

(i) With respect to new claims 584 and 604,¹⁷ it is my opinion and conclusion that the disclosure of Halloran I, taken in further view of Falkow's disclosure (U.S. Patent No. 4, 358,535), would not have rendered the subject matter of these claims obvious to a person of ordinary skill in the art at the time the Engelhardt application was first filed in June 1982. As stated previously, none of Engelhardt's claimed compositions recite or include a polypeptide for the non-radioactive label moiety Sig. In half of the Engelhardt claims, polypeptide is specifically eschewed for the non-radioactive label moiety Sig. Moreover, the Engelhardt claims do not require or include enzymes as a label moiety Sig. Thus, in my opinion and conclusion, Falkow's disclosure with respect to enzymes as labels does not provide the requisite disclosure which is lacking in Halloran I, and which would have been necessary in order to reach the compositions in the Engelhardt application.

¹⁷ New claims 584 and 604 correspond to former claims 462 and 490.

(ii) With respect to new claims 591 and 611,¹⁸ it is my opinion and conclusion that even Falkow's disclosure of fluorescent compounds, combined with Halloran I's disclosure, would not have rendered the Engelhardt compositions obvious to a person of ordinary skill in the art. Again, it is my opinion and conclusion that Halloran I taken in further view of Falkow's patent, would not reach Engelhardt's compositions which either require a non-polypeptide, non-radioactive label moiety Sig, or which do not recite or include a polypeptide for members of Sig, or which include a polypeptide or a protein chemical linkage to attach Sig to the phosphate moiety.

(iii) With respect to new claims 590 and 610,¹⁹ it is my opinion and conclusion that even Falkow's disclosure regarding heavy metals, when combined with Halloran I, would not have rendered Engelhardt's claims obvious to a person of ordinary skill in the art at the time the application was filed in June 1982. As I have stated earlier in this Declaration, Engelhardt's claims either recite a non-polypeptide, non-radioactive label moiety Sig, or they do not recite or include a polypeptide among the members of Sig, except as a chemical linkage for attaching Sig to the phosphate moiety.

(iv) With respect to claims 476-477 and 504-505,²⁰ it is my opinion and conclusion that these claims would not have been obvious to one of ordinary skill in the art at the time the invention was made in view of Halloran I, taken in view of Falkow's disclosure regarding ligands and antiligands as labels. Further, it is my opinion and conclusion that it would not have been obvious to have modified Halloran's oligonucleotides by including Falkow's labels, instead of using a protein, in order to have provided an equally effective compound for nucleic acid detection. As indicated in the background above (Paragraph 10 A), the purpose of our

¹⁸ New claims 591 and 611 correspond to former claims 471 and 499.

¹⁹ New claims 590 and 610 correspond to former claims 470 and 498.

²⁰ Former claims 476-477 and 504-505 do not have counterparts in the new claims. Nevertheless, I am addressing the rejection of these claims in the November 26, 2001 Office Action.

investigation which led to the publication of Halloran I and Halloran II was to prepare nucleotide-protein conjugates for eliciting antibody formation with nucleotide specificity in order to study DNA and RNA structure and autoimmunity. As such, it would not have been obvious to discard the protein disclosed in Halloran II for any label disclosed in Falkow's patent. To substitute a protein in Halloran I would have been irrelevant to the purpose or object of our investigation and the conjugates we had prepared, which was to elicit nucleotide-specific antibody formation so that nucleic acid structures and autoimmunity could be studied. Moreover, as I indicated in the background above, we were not motivated to employ a detection label, let alone a non-radioactive detectable label, because we were only seeking to produce antibody formation with nucleotide specificity.

(D) The Fifth Rejection (Obviousness)

(i) With respect to new claims 625 and 645,²¹ it is my opinion and conclusion that the disclosure of Halloran II, taken in further view of Falkow's disclosure (U.S. Patent No. 4, 358,535), would not have rendered the subject matter of these claims obvious to a person of ordinary skill in the art at the time the Engelhardt application was first filed in June 1982. As I indicated in the preceding section (13(i)), none of Engelhardt's claimed compositions recite or include a polypeptide for the non-radioactive label moiety Sig. In a third of Engelhardt's claims, polypeptide is specifically excluded for the non-radioactive label moiety Sig. Moreover, the Engelhardt claims do not require or include enzymes as a label moiety Sig. Thus, in my opinion and conclusion, Falkow's disclosure with respect to enzymes as labels does not provide the requisite disclosure which is lacking in Halloran II, and which would have been necessary in order to reach the compositions now being claimed in the Engelhardt application.

²¹ New claims 625 and 645 correspond to former claims 519 and 547.

(ii) With respect to new claims 632 and 652,²² it is also my opinion and conclusion that even Falkow's disclosure of fluorescent compounds, combined with Halloran II's disclosure, would not have rendered the Engelhardt compositions obvious to a person of ordinary skill in the art. Again, it is my opinion and conclusion that Halloran II taken in further view of Falkow's patent, would not reach Engelhardt's compositions which either require a non-polypeptide, non-radioactive label moiety Sig, do not recite or include a polypeptide for members of Sig, or that include a polypeptide or a protein as a chemical linkage to attach Sig to the phosphate moiety. It is also my opinion and conclusion that the Falkow patent provides little or no information on the preparation of a fluorescent label, nor its attachment to a nucleotidyl phosphate, as set forth in the Engelhardt claims. Indeed, if Falkow's idea was to label an otherwise unmodified oligonucleotide with fluorescein isothiocyanate, I doubt whether Falkow's preparations would have been useful. Potential problems might very well have included insufficient stability, reduced or relatively non-specific hybridization signal, and especially, inefficient labeling.

(iii) With respect to new claims 631 and 651,²³ it is also my opinion and conclusion that even Falkow's disclosure regarding heavy metals, when combined with Halloran II, would not have rendered Engelhardt's claims obvious to a person of ordinary skill in the art at the time the application was filed in June 1982. As I have stated earlier in this Declaration, Engelhardt's claims either recite a *non-polypeptide*, non-radioactive label moiety Sig, or they do not recite or include a polypeptide among the members of Sig. Furthermore, I find Falkow's disclosure to be lacking or insufficient on how he or they would have used heavy metals for labeling oligo- or polynucleotides.

²² New claims 632 and 652 correspond to former claims 528 and 556.

²³ New claims 631 and 651 correspond to former claims 527 and 555.

(iv) With respect to former claims 533-534 and 561-562,²⁴ it is my opinion and conclusion that these claims would not have been obvious to one of ordinary skill in the art at the time the invention was made in view of Halloran II, taken in view of Falkow's disclosure regarding ligands and antiligands as labels. Further, it is my opinion and conclusion that it would not have been obvious to have modified Halloran's oligonucleotides by including Falkow's labels, instead of using a protein, in order to have provided an equally effective compound for nucleic acid detection. I indicated in the background above (Paragraph 10 A) that the purpose of our investigation which culminated in the publication of Halloran I and Halloran II was to prepare nucleotide-protein conjugates for antibody formation with nucleotide specificity to study DNA and RNA structure and the possible induction of autoimmune disease. As such, it would not have been obvious to discard the protein disclosed in Halloran II for any label disclosed in Falkow's patent. To substitute the protein in Halloran I would have been irrelevant to the purpose or object of our investigation and the conjugates we had prepared, which was to elicit nucleotide-specific antibody formation so that nucleic acid structures and possible consequences of anti-DNA antibody formation could be studied. Moreover, as I indicated in the background above (Paragraph 10 D), we were not motivated or even inclined to employ a detection label, let alone a non-radioactive detectable label, because we were only seeking to produce antibody formation with nucleotide specificity.

(E) The Sixth Rejection (Obviousness)

(i) With respect to new claims 582-583, 587, 588-589, 602, 607 and 608-609,²⁵ it is my opinion that these claims would not have been rendered

²⁴ Former claims 533-534 and 561-562 do not have counterparts in the new claims. The rejection of these claims is being addressed nevertheless.

²⁵ New claims 582-583, 587, 588-589, 602, 607 and 608-609 correspond to former claims 460-461, 465, 467-468, 488, 493 and 495-496.

obvious by Halloran I's disclosure taken in further view of Ward et al. (U.S. Patent No. 4,711,955). As described in the background above (Paragraph 10 B), in Halloran I, we were seeking to couple the nucleotide or polynucleotide directly to the protein, and at the same time, to minimize modifications or changes to the nucleic acid polymer or monomer being coupled. This is described on page 378 (left column) in Halloran I:

In the method of conjugation described in this article, mononucleotides, oligonucleotides, and DNA are coupled directly to protein. By contrast, the methods described in the preceding paragraph require some form of preliminary chemical modification of the mononucleotide, nucleoside or base. The applicability of these methods to coupling large units (oligo- and polynucleotides) to proteins or polyamino acids has not been established. We believe, therefore, that the general method described herein has certain advantages over previously described procedures.

To state it in another way, we were preparing nucleotide-protein conjugates in which the nucleotide or polynucleotide portion of the conjugate was directly coupled to the protein through an easily introduced terminal 5'-phosphate without an additional chemical linkage or linker arm and with minimal changes to the nucleotide or polynucleotide. It would have been irrelevant to Halloran I's disclosure to include any of the chemical linkages disclosed in Ward's patent, particularly since the former was aimed to direct coupling of the nucleotide or polynucleotide to the protein. It is my opinion and conclusion as a person of ordinary skill in the art that it would not have been obvious to modify the direct coupling linkage in Halloran I with any of the chemical linkages disclosed in Ward's patent.

(ii) Regarding any of the labels disclosed in Ward's patent, it is also my opinion and conclusion that it would not have been obvious to a person of ordinary skill in the art to use Ward's disclosed labels (biotin, fluorescent dyes, electron-dense reagents, such as ferritin, colloidal gold and ferric oxide, or enzymes, such as

peroxidase and alkaline phosphatase), instead of the protein in Halloran I. As stated earlier in this Declaration (Paragraph 10 A), the purpose of our investigation which culminated in the publication of Halloran I and Halloran II was to prepare nucleotide-protein conjugates for antibody formation with nucleotide specificity to study DNA and RNA structure. In my opinion as a person of ordinary skill in the art, it would not have been obvious to discard the protein disclosed in Halloran II for any label disclosed in Ward's patent. To substitute a protein in Halloran I would have been irrelevant to and would have negated the very purpose or object of our investigation and the conjugates we had prepared, which was to elicit nucleotide-specific antibody formation so that nucleic acid structures could be studied. Moreover, as indicated in the background above (Paragraph 10 D), we were not motivated or seeking to employ a detection label, let alone a non-radioactive detectable label, because we sought instead to elicit antibody formation with nucleotide specificity. Furthermore, as a person of ordinary skill in the art, I would not have been inclined to look to Ward's patent, which was directed to minimally disruptive base labeling and the incorporation (for example, enzymatically) of such base labeled nucleotides, in order to modify the conjugates in Halloran I, the latter having the protein conjugated to the phosphate of the nucleotide or polynucleotide -- and not to minimally disruptive base positions as in the case of Ward. Sterically, the effects of incorporating signals through the base and phosphate moieties are different and the resulting conjugates or oligonucleotide compositions might well have different sensitivities, selectivities or applications. As an ordinarily skilled person, I consider Ward's procedures to represent a wholly different methodological approach to oligonucleotide detection from Halloran I because Ward emphasizes labels attached through specific base positions. In contrast, the Engelhardt claims are directed to oligo- or polynucleotides in which a non-radioactive label moiety Sig is attached to the phosphate moiety of a modified nucleotide. This attachment runs counter to Ward's "essential criteria" (quoted in

the Eighth Rejection below) and minimally disruptive positions described in their patent.

(F) The Seventh Rejection (Obviousness)

(i) With respect to new claims 623-624, 628, 629-630, 643-644, 648 and 649-650,²⁶ it is my opinion and conclusion that the subject matter of these claims would not have been obvious over Halloran II's disclosure, taken further in view of Ward's U.S. Patent No. 4,711,955. As I indicated in the preceding Paragraph 15(i), we sought to directly couple the nucleotide or polynucleotide to the protein, and to do so with little or no modifications or changes to the ribonucleic or deoxyribonucleic acid polymer or monomer being coupled. These conjugates were used for antibody formation in Halloran II. Thus, the nucleotide-protein conjugates used in Halloran II had the nucleotide or polynucleotide portion of the conjugate directly coupled to the protein without an additional chemical linkage or linker arm and with minimal changes to the nucleotide or polynucleotide. It would have run contrary to the disclosure in Halloran II to include any of the chemical linkages disclosed in Ward's patent, particularly since the former was aimed to produce antibody formation with nucleotide specificity using conjugates in which the nucleotide or polynucleotide was directly coupled to the protein. It is my opinion and conclusion as a person of ordinary skill in the art that it would not have been obvious to modify the direct coupling linkage in Halloran II with any of the chemical linkages disclosed in Ward's patent.

(ii) Regarding any of the labels disclosed in Ward's patent, it is also my opinion and conclusion that it would not have been obvious to a person of ordinary skill in the art to use Ward's disclosed labels (biotin, fluorescent dyes, electron-dense reagents, such as ferritin, colloidal gold and ferric oxide, or enzymes, such as

²⁶ New claims 623-624, 628, 629-630, 643-644, 648 and 649-650 correspond to former claims 517-518, 522, 524-525, 545-546, 550 and 552-553.

peroxidase and alkaline phosphatase), instead of the protein in Halloran II. As stated earlier in this Declaration (Paragraph 10 A), the purpose of our investigation which culminated in the publication of Halloran I and Halloran II was to prepare nucleotide-protein conjugates for antibody formation with nucleotide specificity to study DNA and RNA structure. In my opinion as a person of ordinary skill in the art, it would not have been obvious to discard the protein disclosed and used for antibody formation in Halloran II for any label disclosed in Ward's patent. To substitute a protein in Halloran II's conjugates, would have been irrelevant to and would have negated the purpose or object of our investigation and the conjugates we had prepared, which was to elicit nucleotide-specific antibody formation so that nucleic acid structures could be studied. Moreover, as indicated in the background above (Paragraph 10 D), we were not motivated or even seeking to employ a detection label, let alone a non-radioactive detectable label, because we sought to elicit antibody formation with nucleotide specificity. Furthermore, as a person of ordinary skill in the art, I would not have been inclined to look to Ward's patent, which was directed to minimally disruptive base labeling, in order to modify the conjugates in Halloran II, the latter having the protein conjugated to the *phosphate* of the nucleotide or polynucleotide -- and *not* to the base as in the case of Ward's labels.

(G) The Eighth Rejection (Obviousness)

(i) With respect to former claims 475 and 503,²⁷ it is my opinion and conclusion that the subject matter of these claims would not have been obvious to a person of ordinary skill in the art from a reading of Ward's patent, taken in further view of Halloran I. More particularly, it would not have been obvious in view of

²⁷ Former claims 475 and 503 do not have counterparts in the new claims. I am addressing the rejection insofar as it may be applicable to new claims 736-813 and the claimed embodiment wherein a non-radioactive label moiety Sig is covalently attached to the phosphate moiety through a chemical linkage comprising a polypeptide or a protein.

Dean L. Engelhardt et al.

Serial No. 08/479,997

Filed: June 7, 1995

Page 34 [Declaration of Dr. Charles W. Parker]

Halloran I's disclosure of coupling proteins, such as HSA, B₇G and polylysine, which can be stained with Amidoschwartz, to have modified Ward's compounds to include polylysine (instead of rhodamine) so as to have achieved an equally effective compound for nucleic acid detection. As stated above in several paragraphs, Amidoschwartz and Feulgen stains are crude, non-specific stains and could not have been used for effective and accurate signaling from a hybridized nucleic acid duplex. I respectfully point out that Ward's nucleic acid compounds include base-modified nucleotides in which the base has been modified in the so-called "minimally disruptive" positions. That this is the case is spelled out in clear language in Ward's patent, beginning in the section "Detailed Description of the Invention," column 6, line 36, through column 7, line 17:

Several essential criteria must be satisfied in order for a modified nucleotide to be generally suitable as a substitute for a radioactively-labeled form of a naturally occurring nucleotide. First, the modified compound must contain a substituent or probe that is unique, i.e., not normally found associated with nucleotides or polynucleotides. Second, the probe must react specifically with chemical or biological reagents to provide a sensitive detection system. Third, the analogs must be relatively efficient substrates for commonly studied nucleic acid enzymes, since numerous practical applications require that the analog be enzymatically metabolized, e.g., the analogs must function as substrates for nucleic acid polymerases. For this purpose, probe moieties should not be placed on ring positions that sterically, or otherwise, interfere with the normal Watson-Crick hydrogen bonding potential of the bases. Otherwise, the substituents will yield compounds that are inactive as polymerase substrates. Substitution at ring positions that alter the normal "anti" nucleoside conformation also must be avoided since such conformational changes usually render nucleotide derivatives unacceptable as polymerase substrates. Normally, such considerations limit substitution positions to the 5-position of a pyrimidine and the 7-position of a purine or a 7-deazapurine.

Fourth, the detection system should be capable of interacting with probe substituents incorporated into both single-stranded and double-stranded polynucleotides in order to be compatible with nucleic acid hybridization methodologies. To satisfy this criterion, it is

preferable that the probe moiety be attached to the purine or pyrimidine through a chemical linkage or "linker arm" so that it can readily interact with antibodies, other detector proteins, or chemical reagents.

Fifth, the physical and biochemical properties of polynucleotides containing small numbers of probe substituents should not be significantly altered so that current procedures using radioactive hybridization probes need not be extensively modified. This criterion must be satisfied whether the probe is introduced by enzymatic or direct chemical means.

Finally, the linkage that attaches the probe moiety should withstand all experimental conditions to which normal nucleotides and polynucleotides are routinely subjected, e.g., extended hybridization times at elevated temperatures, phenol and organic solvent extraction, electrophoresis, etc.

All of these criteria are satisfied by the modified nucleotides described herein.

(ii) It is my opinion and conclusion that a person of ordinary skill in the art would not have looked to Halloran I to modify Ward's compounds by including proteins or polypeptides (such as HSA and poly-lysine) and protein stains, notably Amidoschwartz. As indicated in several preceding paragraphs above, the use of Amidoschwartz as a crude, non-quantitative staining technique in no way points to its use for detecting signals from hybridized nucleic acid duplexes. As clearly indicated in the portion of Ward et al. just quoted above, it teaches away from Ward's patent to modify the Ward compounds by coupling Halloran I's proteins to the phosphate moiety. The phosphate moiety is not at a minimally disruptive base position as urged and required in Ward's above-quoted passage. Furthermore, it is my opinion and conclusion that even if a person of ordinary skill in the art were to include Halloran I's proteins in Ward's compounds, he or she would have been motivated to attach the protein only to the minimally disruptive base position, such as the 5-position of a pyrimidine, the 8-position of a purine, or the 7-position of a deazapurine. To do otherwise would require a repudiation of Ward's "several

essential criteria" quoted above.

(H) The Ninth Rejection (Obviousness)

(i) With respect to claims 532 and 560,²⁸ it is also my opinion and conclusion that it would not have been obvious to one of ordinary skill in the art at the time the Engelhardt application was filed in June 1982 to have modified Ward's compound to include Halloran II's polylysine as a detectable label (instead of rhodamine) so as to have achieved an equally effective compound for nucleic acid detection. As I indicated in the preceding discussion for the seventh rejection (Paragraph 16(i)), it would not have been obvious in view of Halloran II's disclosure which calls for coupling proteins directly to the phosphate moiety of a mononucleotide or polynucleotide, to have modified Ward's compounds to include polylysine (instead of rhodamine) so as to have achieved an equally effective compound for nucleic acid detection. As indicated in the preceding discussion (Paragraphs 17(i) and 17(ii)), Ward's nucleic acid compounds include base-modified nucleotides in which the base has been modified in the so-called "minimally disruptive" positions. See Ward et al., U.S. Patent No. 4,711,955, under the "Detailed Description of the Invention," column 6, line 36, through column 7, line 17; quoted in the preceding section (seventh rejection). The phosphate moiety employed in Halloran II's conjugates is not at a minimally disruptive base position as urged and required in Ward's passage quoted in the preceding discussion for the seventh rejection. Furthermore, it is my opinion and conclusion that even if a person of ordinary skill in the art were to include Halloran I's polylysine polypeptide in Ward's compounds, at most he or she would have been motivated to attach the protein to the minimally disruptive base position in Ward's compound, which is limited to the 5-position of a pyrimidine, the 8-position of a purine, or the 7-position

²⁸ Although they do not have counterparts in the new claims, the rejection of former claims 532 and 560 is being addressed nevertheless.

Dean L. Engelhardt et al.
Serial No. 08/479,997
Filed: June 7, 1995
Page 37 [Declaration of Dr. Charles W. Parker]

of a deazapurine. To do otherwise and attach the polylysine to the phosphate moiety would require repudiation of Ward's "several essential criteria" and a clear teaching away from Ward's patent.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that any such willful false statements may jeopardize the validity of the application or any patent issued thereon.

5/21/02
Date

Charles W. Parker
Charles W. Parker, M.D.

* * * * *

Enz-5(D6)(C2).FinalDecl.CWP.5.20.02

CURRICULUM VITAE -Charles W. Parker, M.D.

SS#: 490-38-0488

Date: October 6, 1999

1. Personal Information:
 - a. Sex: M
 - b. Date of Birth: March 23, 1930
 - c. Place of Birth: St. Louis, Missouri
2. Citizenship: USA
3. Address and Telephone Numbers:
 - a. , St. Louis, Missouri 63124
 - b. Home: (314) 962-9250
4. Present Position:

Professor of Medicine, Microbiology and Immunology (*Emeritus*)
5. Education:
 - a. Washington University, St. Louis, MO 1947-1949
 - b. Washington University School of Medicine, St. Louis, MO, 1958
 - c. 1953-1954 Intern, Barnes Hospital, Washington University School of Medicine, St. Louis, MO
 - 1956-1958 Assistant Resident, Barnes Hospital, Washington University School of Medicine, St. Louis, MO
 - 1958-1959 Chief Resident, Barnes Hospital (Ward Medical Service), Washington University School of Medicine, St. Louis, MO
 - 1961-1962 USPHS Research Fellow
 - 1962-1972 Research Career Award, NIAID
6. Academic Positions/Employment:

1960-1963	Instructor in Medicine, Washington University School of Medicine
1962-1988	Head, Division of Allergy and Immunology, Washington University School of Medicine
1963-1968	Assistant Professor of Medicine, Washington University School of Medicine
1968-1971	Associate Professor of Medicine, Washington University School of Medicine
1971-1998	Professor of Medicine, Washington University School of Medicine
1975-1998	Professor of Microbiology and Immunology Molecular B, Washington University School of Medicine
1977-1989	Investigator, Howard Hughes Laboratory for the Study of Clinical Immunology and Allergy at Washington University School of Medicine
1998-	Professor of Medicine (<i>Emeritus</i>), Washington University School of Medicine
7. University and Hospital Appointments and Committees:

1959-1998	Associate Physician, Barnes Hospital
-----------	--------------------------------------
8. Medical Licensure

MO 15764; Board Certified Internal Medicine, 1962

9. Military Service:
LCMR, USNR United States Navy; 1954-1956
10. Honors and Awards:
Washington University, cum laude
Mosby Award, Phi Eta Sigma; Alpha Omega Alpha; Sigma Xi; Hixon Award; Bausch and Lomb Award
Research Career Award, NIAID, 1962-1972
Honorary Fellowship Award, The American Academy of Allergy and Immunology, 1983 (for original research in the field of allergy and immunology)
Washington University Alumni Award
Charles W. Parker Medical Student Scholarship
11. Editorial Responsibilities: (Editorial Boards)
Journal of Allergy and Clinical Immunology
Immunochemistry
Clinical Immunology and Immunopathology
Journal of Immunology
Journal of Clinical Investigation
Editorial Consultant to Clinical Immunology
Editorial Committee, American Association of Immunologists
Associate Editor, The Journal of Clinical Investigation, 1977-1982
Section Editor, The Journal of Immunology, 1977-1982
12. Professional Societies and Organizations:
American Board of Internal Medicine
Central Society for Clinical Research
Fellow, American Academy of Allergy
American Association of Immunologists
Collegium Internationale Allergologicum
Association of American Physicians
American Heart Study Section, 1972-1974
American Society for Clinical Investigation
Council of the American Society for Clinical Investigation, 1973-1976
American Federation for Clinical Research
13. Major Invited Professorships and Lectureships:
Various lectureships and symposia.
14. Consulting Relationships and Board Memberships
NIH Study Section - Immunology A, 1967-1971
FDA Anti-infective Agents Advisory Committee, 1969-1971
Consultant to NIH Intramural Training Programs in Clinical Immunology
NIH, NIAID, Allergy and Immunology Research and Research Training Committee, 1972-1974
Board of Advisors, Roche Institute of Molecular Biology, 1978-1981
Ad hoc Consultant to numerous pharmaceutical companies including Searle, Merck, Pfizer, Lilly, Mead Johnson, Nippon Zoki
NIH, GCRC Scientific Review Committee, 1993-1997, 1998-2002, *ad hoc reviews*

15. Research Support: (Current)
Retired

16. Clinical Title and Responsibilities:
1959-1998 Associate Physician, Barnes-Jewish Hospital

17. Teaching Title and Responsibilities:
1971-1998 Professor of Medicine, Washington University School of Medicine
1975-1998 Professor of Microbiology and Immunology, Molecular Biology, Washington University School of Medicine
1998- Professor of Medicine (*Emeritus*), Washington University School of Medicine

18. Bibliography

(a) Manuscripts

1. Velick, SF, Parker, CW, and Eisen, HN. Excitation energy transfer and the quantitative study of the antibody hapten reaction. *Proc Natl Acad Sci* 1960; 46:1470.
2. Parker, CW, Kern, M and Eisen, HN. Polyfunctional dinitrophenyl haptens as reagents for elicitation of immediate-type allergic skin responses. *J Exp Med* 1962; 115:789.
3. Parker, CW, DeWeck, AL, Kern, M and Eisen, HM. The preparation and some properties of penicillinic acid derivatives relevant to penicillin hypersensitivity. *J Exp Med* 1962; 115:803.
4. Parker, CW, Shapiro, J, Kern, M, and Eisen, HM: Hypersensitivity to penicillinic acid derivatives in humans with penicillin allergy. *J Exp Med* 1962; 115:831.
5. Parker, CW: Editorial - Penicillin Allergy. *Amer J Med* 1963; 34:747.
6. Parker, CW and Thiel, JA. Studies in human penicillin allergy. A comparison of various penicilloyl-polylysines. *J Lab Clin Med* 1963; 62:482.
7. Parker, CW. The immunochemical basis for penicillin allergy. *Postgraduate Med J* 1964; 40:141.
8. Budd, MA, Parker, CW and Norden, CW. Evaluation of intradermal skin tests in penicillin hypersensitivity. *JAMA* 1964; 190:203.
9. Thiel, JA, Mitchel, S and Parker, CW. The specificity of hemagglutination reactions in human and experimental penicillin hypersensitivity. *J Allergy* 1964; 35:399.
10. Parker, CW, Thiel, JA and Mitchell, S. The immunogenicity of hapten-polylysine conjugates. *J Immunol* 1964; 94:289.
11. Parker, CW. The biochemical basis of an allergic drug response. Symposium on Drug Toxicity. *Ann NY Acad Sci* 1964; 125:55.
12. Parker, CW. Immunochemical mechanisms in penicillin allergy. Symposium on Allergic Responses to Pharmacologic Agents. *Fed Proc* 1965; 24:51.
13. Halloran, MJ and Parker, CW. The preparation of nucleotide-protein conjugates. Carbodiimides as coupling agents. *J Immunol* 1966; 96:373.
14. Halloran, MJ and Parker, CW. The production of antibodies to mononucleotides, oligonucleotides and DNA. *J Immunol* 1966; 96:379.
15. Permutt, A, Parker, CW and Utiger, RD. Studies on the immunochemistry of lysine vasopressin and other posterior pituitary hormones. *Endocrinology* 1966; 78:809.
16. Parker, CW, Bodt, S and Johnson, MC. The antibody response to a 2,4-dinitrophenyl peptide. *Biochemistry* 1966; 5:2314.
17. Parker, CW, Yoo, TJ, Johnson, MC and Godt, SM. Fluorescent probes for the study of the antibody hapten reaction. I. Binding of the 5-dimethylaminonaphthalene-1-sulfonamido group by homologous rabbit antibody. *Biochemistry* 1967; 6:3408.

18. Parker, CW, Godt, S and Johnson, MC. Fluorescent probes for the study of the antibody hapten reaction. II. Variation in the antibody combining site during the immune response. *Biochemistry* 1967; 6:3417.
19. Parker, CW. Enterococcal endocarditis. Allergy to penicillin. Jewish Hospital (St. Louis) Grand Rounds. *JAMA* 1968; 204:916.
20. Yoo, TJ and Parker, CW. Fluorescent enhancement in antibody hapten interaction 1-anilinonaphthalene-8-sulfonate as a fluorescent probe at active sites. *Immunochemistry* 1968; 5:143.
21. Oliver, GC, Parker, BM, Brasfield, DL and Parker, CW. The measurement of digitoxin in human serum by radioimmunoassay. *J Clin Invest* 1968; 47:1035.
22. Dorner, IM, Parker, CW and Chaplin, H. Autoagglutination developing in a patient with acute renal failure. *Brit J Hematology* 1968; 14:379.
23. Ljaljevic, M, Ljaljevic, J and Parker, CW. Modification of the amino groups of rabbit (-G globulin. I. The effect on skin sensitizing activity in guinea pig skin. *J Immunol* 1968; 100:1041.
24. Ljaljevic, M, Ljaljevic, J and Parker, CW. Modification of the amino groups of rabbit (-G globulin. II. Selective effect on Fc function. *J Immunol* 1968; 100:1051.
25. Steiner, AL, Kipnis, DM, Utiger, R and Parker, CW. Radioimmunoassay for the measurement of adenosine 3', 5' cyclic monophosphate. *Proc Natl Acad Sci* 1969; 64: 367.
26. Messner, RP, Parker, CW and Williams, RC. Chemical modification of human and rabbit anti-staphylococcal (-G antibodies. Effect of acetylation, carbamylation and amidation on opsonic activity. *J Immunol* 1970; 104: 238.
27. Parker, CW and Osterland, CK. Hydrophobic binding sites on immunoglobulins. *Biochemistry* 1970; 9:1074.
28. Spector, S and Parker, CW. Morphine: Radioimmunoassay. *Science* 1970; 168: 1347.
29. Fisher, BD, Parker, CW, Haynes, CR and Chaplin, H. Inhibition of erythrocyte antibody binding by a dialysate of hemolyzed red blood cells. *Clin Chem Acta* 1970; 30:842.
30. Oliver, GC, Parker, CM and Parker, CW. Radioimmunoassay for dioxin. Technique and Clinical application. *Amer J Med* 1971; 51:186.
31. Smith, JW, Steiner, AL, Newberry, WM and Parker, CW. Cyclic AMP in human lymphocytes. Alterations following phytohemagglutinin stimulation. *J Clin Invest* 1971; 50:432.
32. Smith, JW, Steiner, AL and Parker, CW. Human lymphocyte metabolism. Effects of cyclic and noncyclic nucleotides on stimulation by phytohemagglutinin. *J Clin Invest* 1971; 50:442.
33. Parker, CW, Smith, JW and Steiner, AL. Early effects of phytohemagglutinin (PHA) on lymphocyte cyclic AMP levels. *Int Arch Allergy* 1971; 41:40.
34. Jaffe, BM, Smith, JW, Newton, WT and Parker, CW. Radioimmunoassay for prostaglandins. *Science* 1971; 171:494.
35. Aach, RD, Grisham, JW and Parker, CW. Detection of Australia antigen by radioimmunoassay. *Proc Natl Acad Sci* 1971; 68:1056.
36. Liskow, B, Liss, JL and Parker, CW. Allergy to marijuana. A case report. *Ann of Int Med* 1971; 75:571.
37. Jaffe, BM, Parker, CW and Philpott, GW. Immunochemical measurement of prostaglandin or prostaglandin-like activity from normal and neoplastic tissues in culture. *Surgical Forum* 1971; 22:90.
38. Stein, MR and Parker, CW. Reactions following intravenous fluorescein. *Amer J Ophthalmol* 1971; 72: 861.
39. Oliver, GC, Bahl, OP and Parker, CW. Characterization of antialdosterone antibodies. *Circulation* 1971; 44:446.
40. Wedner, HJ, Hoffer, BJ, Battenberg, E, Steiner, AL, Parker, CW, and Bloom, FE. A method for detecting cyclic AMP by immunofluorescence. *J Histochem Cytochem* 1972; 20:293.

41. Eisen, SA, Wedner, HJ and Parker, CW. Isolation of pure human peripheral T lymphocytes on nylon columns. *Immunologic Comm* 1972; 1:571.
42. Bloom, FE, Hoffer, BJ, Battenberg, ER, Siggins, GR, Steiner, AL, Parker, CW and Wedner, HJ. Adenosine 3', 5' monophosphate is localized in cerebellar neuron. Immunofluorescence evidence. *Science* 1972; 177:436.
43. Steiner, AL, Parker, CW and Kipnis, DM. Radioimmunoassay for cyclic nucleotides. I. Preparation of antibodies and iodinated cyclic nucleotides. *J Biol Chem* 1972; 247:1106.
44. Ryan, JJ, Parker, CW and Williams, RC. (- Globulin binding of morphine in heroine addicts. *J Lab Clin Med* 1972; 80:155.
45. Steiner, AL, Wehmann, RE, Parker, CW and Kipnis, DM. Radioimmunoassay for the measurement of cyclic nucleotides. *Adv Cyclic Nucleotide Res* 1972; 2:51.
46. Jaffe, BM, Parker, CW, Marshall, GR and Needleman, P. Renal concentrations of prostaglandin E in acute and chronic renal ischemia. *Biochem Biophys Res Commun* 1972; 49:799.
47. Parker, CW and Smith, JW. Alterations in cyclic AMP metabolism in human bronchial asthma. I. Leukocyte responsiveness in β adrenergic agents. *J Clin Invest* 1973; 52:48.
48. Jaffe, BM and Parker, CW. Physiological implications of prostaglandin radioimmunoassay. *Metabolism* 1973; 22:8.
49. Jaffe, BM, Behrman, HR and Parker, CW. Radioimmunoassay measurement of prostaglandins E, A and F in human plasma. *J Clin Invest* 1973; 52:398.
50. Parker, CW and Morse, SI. The effect of *B. pertussis* on lymphocyte cyclic AMP metabolism. *J Exp Med* 1973; 137:1078.
51. Knowles, DM, Sullivan, TJ, Parker, CW and Williams, RC. *In vitro* antibody-enzyme conjugates with specific bactericidal activity. *J Clin Invest* 1973; 52:1443.
52. Parker, CW. The immunotherapy of cancer. *Pharm Rev* 1973; 25:325.
53. Eisen, SA, Lyle, LR and Parker, CW. Antigen stimulation of ^3H thymidine incorporation by subpopulations of human peripheral blood cells. *J Immunol* 1973; 111:962.
54. Sullivan, TJ, Parker, KL and Parker, CW. Specific killing of parasites by antibody-enzyme conjugates. *Res Comm Chem Pathol Pharm* 1973; 6:709.
55. Parker, CW and Snider, DE. Prostaglandins and asthma. Editorial. *Ann Int Med* 1973; 78:963.
56. Parker, CW, Baumann, ML and Huber, MG. Alterations in cyclic AMP metabolism in human bronchial asthma. II. Leukocyte and lymphocyte responses to prostaglandin. *J Clin Invest* 1973; 52:1336.
57. Parker, CW, Huber, MG and Baumann, ML. Alterations in cyclic AMP metabolism in human bronchial asthma. III. Leukocyte and lymphocyte responses to steroids. *J Clin Invest* 1973; 52:1342.
58. Philpott, GW, Bower, RJ and Parker, CW. Improved selective cytotoxicity with an antibody diphtheria toxin conjugate. *Surgery* 1973; 73:928.
59. Aach, RD, Hacker, EJ and Parker, CW. Recognition of hepatitis B antigen determinants by a double label radioimmunoassay. A selective means of subtyping hepatitis B antigen. *J Immunol* 1973; 111:381.
60. Philpott, GW, Bower, RJ and Parker, CW. Selective iodination and cytotoxicity *in vitro* and *in vivo* with an antibody enzyme conjugate. *Surgery* 1973; 74:51.
61. Philpott, GW, Bower, RJ and Parker, CW. Selective cytotoxicity in a phaten substituted cell culture model system. *J Immunol* 1973; 111:930.
62. Philpott, GW, Shearer, WT, Bower, RJ and Parker, CW. Selective cytotoxicity of hapten substituted cells with an antibody enzyme conjugate. *J Immunol* 1973; 111:921.
63. Bloom, FE, Wedner, HJ and Parker, CW. The use of antibodies of study cell structure and metabolism. *Pharm Rev* 1973; 25:343.
64. Chao, HF, Peiper, SC, Aach, RD and Parker, CW. Introduction of cellular immunity to a chemically altered tumor antigen. *J Immunol* 1973; 111:1800.

65. Lyle, LR, Eisen, SA and Parker, CW. Mixed leukocyte culture reactions in mouse thymus cells. *Immunol Comm* 1973; 2:255.
66. Shearer, WT, Philpott, GW and Parker, CW. Stimulation of cells by antibody. *Science* 1973; 182:1357.
67. Sullivan, TJ and Parker, CW. Cyclic AMP phosphodiesterase activation by the histamine releasing agent, Compound 48/80. *Biochem Biophys Res Comm* 1973; 55:1334.
68. Shearer, WT, Philpott, GW and Parker, CW. Humoral immunostimulation. I. Increased uptake of ¹²⁵I-iododeoxyuridine and ³H thymide into TNP-cells treated with anti-TNP antibody. *J Exp Med* 1974; 139:367.
69. Lyle, LR and Parker, CW. New approaches to immunosuppression. *Fed Proc* 1974; 33:1889.
70. Pan, P, Bonner, J, Wedner, HJ and Parker, CW. Immunofluorescence evidence for the distribution of cyclic AMP in cells and cell masses of the cellular slime molds. *Proc Natl Acad Sci* 1974; 71:1625.
71. Parker, CW. Unsuspected and uncommon reactions to drugs. Reactions to heroine, aspirin, hydralazine and dextran. *Proc Internat Congress of Immunol Excerpta Medica*, 1974; pp. 426-431.
72. Parker, CW. Cyclic AMP and the β adrenergic system in bronchial asthma. *Proc Internat Congress of Immunol Excerpta Media* 1974; pp. 334-337.
73. Lyle, LR and Parker, CW. The antigen induced proliferative response of murine thymus cells in vitro. *Infection and Immunity* 1974; 10:765.
74. Lyle, LR, Parker, BM and Parker, CW. The use of protein substituted nylon catheters for selective immunoadsorption in vivo. *J Immunol* 1974; 113:517.
75. Shearer, WT, Turnbaugh, TR, Coleman, We, Aach, RD, Philpott, GW and Parker, CW. Cytotoxicity with antibody-glucose oxidase conjugates specific for a human colonic cancer and carcinoembryonic antigen. *Intern J Ca* 1974; 14:539.
76. Philpott, GW, Bower, RJ, Parker, K, Shearer, WT and Parker, CW. Affinity cytotoxicity of tumor cells with antibody-glucose oxidase conjugates, peroxidase and arspenamine. *Ca Res* 1974; 34:2159.
77. Chao, HF, Peiper, SC, Philpott, GW, Parker, CW and Aach, RD. Selective uptake of specifically purified antibodies to CEA by human adenocarcinoma. *Res Comm Chem Path Pharm* 1974; 9:749.
78. Parker, CW. Correlation between mitogenicity and stimulation of calcium uptake in human lymphocytes. *Biochem Biophys Res Comm* 1974; 61:1180.
79. Lyle, LR and Parker, CW. Cyclic AMP responses to concanavalin A in human lymphocytes. Evidence that the response involves specific carbohydrate receptors on the cell surface. *Biochemistry* 1974; 13:5415.
80. Parker, CW, Aach, RD and Philpott, GW. Enzymatic activation and trapping of luminol substituted peptides and proteins. A possible means of amplifying the cytotoxicity of antitumor antibodies. *Proc Natl Acad Sci* 1975; 72:338.
81. Wedner, HJ and Parker, CW. Protein phosphorylation in human peripheral lymphocytes. Stimulation by phytohemagglutinin and N⁶ monobutyl cyclic AMP. *Biochem Biophys Res Comm* 1975; 62:808.
82. Parker, CW. Drug allergy. (Review in three parts). *New Eng J Med* 1975; 292:511, 732, 957.
83. Sullivan, TJ, Parker, KL, Stenson, W and Parker, CW. Modulation of cyclic AMP in purified rat mast cells. I. Responses to pharmacologic, metabolic and physical stimuli. *J Immunol* 1975; 114:1473.
84. Sullivan, TJ, Parker, KL, Eisen, SA and Parker, CW. Modulation of cyclic AMP purified rat mast cells. II. Studies on the relationship between intracellular cyclic AMP concentrations and histamine release. *J Immunol* 1975; 114:1480.
85. Shearer, WT, Atkinson, JP, Frank, MM and Parker, CW. Humoral immunostimulation. IV. Role of complement. *J Exp Med* 1975; 141:736.

86. Shearer, WT, Philpott, GW and Parker, CW. Humoral immunostimulation. II. Increased Nucleoside incorporation, DNA synthesis, and cell growth of L cells treated with anti-L cell antibody. *Cell Immunol* 1975; 17:447.
87. Sullivan, TJ, Greene, WC and Parker, CW. Concanavalin A induced histamine release from normal rat mast cells. *J Immunol* 1975; 115:613.
88. Shearer, WT, Parker, CW. Humoral immunostimulation. III. Requirements for divalent antibody and cellular aggregation. *J Immunol* 1975; 115:278.
89. Shearer, WT, Philpott, GW and Parker, CW. Alteration of L cell growth by antibody-stimulated incorporation of radioactive nucleosides. *Res Comm Chem Path Pharm* 1975; 11:303.
90. Fink, MF, Parker, CW and Shearer, WT. Antibody stimulation of tumor growth in T-cell depleted mice. *Nature* 1975; 255:404.
91. Wedner, HJ, Dankner, R and Parker, CW. Cyclic GMP and lectin induced lymphocyte activation. *J Immunol* 1975; 115:1682.
92. Greene, WC and Parker, CW. A role for cytochalasin-sensitive protein in the regulation of calcium. *Biochem Biophys Res Comm* 1975; 65:456.
93. Atkinson, JP, Wedner, HJ and Parker, CW. Two novel stimuli of cyclic adenosine 3', 5' monophosphate (cAMP) in human lymphocytes. *J Immunol* 1975; 115:1023.
94. Shearer, WT and Parker, CW. Humoral immunostimulation. V. Selection of variant cell lines. *J Exp Med* 1975; 142:1133.
95. Parker, CW, Dankner, RE, Falkenheim, SF and Green, WC. Suggestive evidence for both stimulatory and inhibitory domains on human lymphocytes as indicated by phospholipid turnover studies with wheat germ agglutinin and other lectins. Possible relationship to immune tolerance and malignant transformation. *Immunol Comm* 1976; 5:13.
96. Roberts, R, Sobel, BE and Parker, CW. Radioimmunoassay for creatine kinase isoenzymes. *Science* 1976; 194:855.
97. Parker, CW. The role of cyclic AMP in immunological inflammation. *J Invest Derm* 1976; 67:638.
98. Greene, WC, Parker, CM and Parker, CW. Opposing effects of mitogenic and non-mitogenic lectins on lymphocyte activation. Evidence that wheat germ agglutinin produces a negative signal. *J Biol Chem* 1976; 251:4017.
99. Kulczycki, A, McNearney, TA and Parker, CW. The rat basophilic leukemia cell receptor for IgE. I. Characterization as a glycoprotein. *J Immunol* 1976; 117:661.
100. Sullivan, TJ, Parker, KL, Kulczycki, A and Parker, CW. Modulation of cyclic AMP in purified rat mast cells. III. Studies on the effects of anti-IgE and concanavalin A on cyclic AMP concentration during histamine release. *J Immunol* 1976; 117:713.
101. Greene, WC, Parker, CM and Parker, CW. Calcium and lymphocyte activation. *Cell Immunol* 1976; 25:74.
102. Shearer, WT, Atkins, JP and Parker, CW. Humoral immunostimulation. VI. Increased calcium uptake by cells treated with antibody and complement. *J Immunol* 1976; 117:973.
103. Greene, WC, Parker, CM, and Parker, CW. Colchicine sensitive structures and lymphocyte activation. *J Immunol* 1976; 117:1015.
104. Sullivan, TJ, Parker, CM and Parker, CW. Pharmacological modulation of the inflammatory mediator release by rat mast cells. Federal Symposium. *Amer J Path* 1976; 85:437.
105. Greene, WC, Parker, CM and Parker, CW. Cytochalasin sensitive structures and lymphocyte activation. *Exp Cell Res* 1976; 103:109.
106. Atkinson, JP, Udey, MC, Wedner, HJ and Parker, CW. Studies on the stimulation of cAMP metabolism by heparin solutions containing benzyl alcohol. *J Cyclic Nucleotide Res* 1976; 2:197.

107. Atkinson, JP, Greene, WC, McNearney, TA and Parker, CW. Studies on the stimulation of cyclic adenosine 3', 5' monophosphate (cAMP) metabolism in human lymphocytes by latex polymers. *Exp Cell Res* 1976; 99:395.
108. Snider, DE and Parker, CW. Aspirin effects on lymphocyte cyclic AMP levels in normal human subjects. *J Clin Invest* 1976; 58:524.
109. Parker, CW. Control of lymphocyte function. (Review of Lymphocyte Regulation). *New Eng J Med* 1976; 195:1180.
110. Parker, CW. Symposium on the effects of the environment on the immune system. *Fed Proceed* 1977; 36:1725.
111. Parker, CW. Symposium on the immune responses to environmental antigens absorbed through the gastrointestinal tract. *Fed Proceed* 1977; 36:1732.
112. Snider, DE and Parker, CW. Adenylate cyclase activity in lymphocyte subcellular fractions. Characterization of non-nuclear adenylate cyclase. *Biochem J* 1977; 162:473.
113. Wedner, HJ and Parker, CW. Adenylate cyclase activity in lymphocyte subcellular fractions. Characterization of a nuclear adenylate cyclase. *Biochem J* 1977; 162:483.
114. Jakschik, BA, Kulczycki, A, MacDonald, HH and Parker, CW. Release of slow reacting substance (SRS) from rat basophilic leukemia (RBL-1) cells. *J Immunol* 1977; 119:618.
115. Atkinson, JP, Michael, JM, Chaplin, H and Parker, CW. Modulation of macrophage C3b receptor function by cytochalasin sensitive structures. *J Immunol* 1977; 118:1292.
116. Kelly, JP, Wedner, HJ and Parker, CW. Mitogenic receptors on human peripheral blood lymphocytes. The interaction of Phaseolus vulgaris (E-PHA) and ATG on the human peripheral blood lymphocytes. *J Immunol* 1977; 118:2213.
117. Parker, CW, Kernnedy, S and Eisen, AZ. Leukocyte and lymphocyte cyclic AMP responses in atopic eczema. *J Invest Dermatol* 1977; 68:302.
118. Atkinson, JP, Sullivan, TJ, Kelly, JP and Parker, CW. Stimulation by alcohols of cAMP metabolism in human lymphocytes. Possible role of cAMP in the anti-inflammatory effects of ethanol. *J Clin Invest* 1977; 60:284.
119. Atkinson, JP and Parker, CW. Modulation of macrophage Fc receptor function by cytochalasin-sensitive structures. *Cell Immunol* 1977; 33:353.
120. Jakschik, BA, Falkenheim, S and Parker, CW. Precursor role of arachidonic acid in slow reacting substance release from rat basophilic leukemia cells. *Proc Natl Acad Sci USA* 1977; 74:4577.
121. Roberts, R, Parker, CW and Sobel, BE. Detection of acute myocardial infarction with a radioimmunoassay for MB creatine kinase. *Lancet* 1977; 2:319.
122. Shearer, WT and Parker, CW. Antibody and complement modulation of tumor cell growth in vitro and in vivo. *Fed Symposium Fed Proceed* 1978; 37:2385.
123. Atkinson, JP, Kelly, JP, Weiss, A, Wedner, HJ and Parker, CW. Enhanced intracellular cGMP concentrations and lectin-induced lymphocyte transformation. *J Immunol* 1978; 121:2282.
124. Parker, CW, Levitt, RG, Wood, J, King, D, Stenson, B, Cryer, P and Herbold, D. Persistent emphysema, bacteremia and death. *Amer J Med* 1978; 64:161.
125. Parker, CW. Cyclic AMP and lectin-induced mitogenesis in lymphocytes. Possible implications for neoplastic cell growth. *Adv Cyclic Nucleotide Res* 1978; 9:647.
126. Marquardt, DL, Parker, CW and Sullivan, TJ. Potentiation of mast cell mediator release by adenosine. *J Immunol* 1978; 120:871.
127. Roberts, R, Sobel, BE and Parker, CW. Immunologic detection of myocardial infarction with a radioimmunoassay for MB creatine kinase. *Clin Chim Acta* 1978; 83:141.
128. Jakschik, BA, Lee, LN, Shuffer, G and Parker, CW. Arachidonic acid metabolism in rat basophilic leukemia (RBL-1) cells. *Prostaglandins* 1978; 16:733.
129. Atkins, JP and Parker, CW. Cytochalasin binding to macrophages. *Cell Immunol* 1978; 41:103.
130. Parker, CW. Prostaglandins and slow reacting substance. *J Allergy and Clin Immunol* 1979; 63:1.

131. Kulczycki, A Jr. and Parker, CW. The cell-surface receptor for immunoglobulin E. I. The use of repetitive affinity chromatography for the purification of a mammalian receptor. *J Biol Chem* 1979; 254:3194.
132. Kulczycki, A Jr., Hempstead, BL, Hofmann, SL, Wood, EW and Parker, CW. The cell-surface receptor for immunoglobulin E. II. Properties of the purified biologically-active receptor. *J Biol Chem* 1979; 254:3194.
133. Kennerly, DA, Sullivan, TJ and Parker, CW. Activation of phospholipid metabolism during mediator release from stimulated rat mast cells. *J Immunol* 1979; 122:152.
134. Sullivan, TJ and Parker, CW. Possible role of arachidonic acid and its metabolites in mediator release from rat mast cells. *J Immunol* 1979; 122:431.
135. Yecies, LD and Parker, CW. Slow reacting substance (SRS) biosynthesis, characterization and purification. *Monograph of Allergy* 1979; 14:155.
136. Kelly, JP and Parker, CW. Effects of arachidonic acid and other unsaturated fatty acids on mitogenesis in human lymphocytes. *J Immunol* 1979; 122:1556.
137. Kelly, JP, Johnson, MC and Parker, CW. Effect of inhibitors of arachidonic acid metabolism of mitogenesis in human lymphocytes. Possible role of thromboxanes and products of the lipoxygenase pathway. *J Immunol* 1979; 122:1563.
138. Parker, CW, Stenson, WF, Huber, MG and Kelly, JP. Formation of thromboxane B₂ and hydroxyarachidonic acids in purified human lymphocytes in the presence and absence of PHA. *J Immunol* 1979; 122:1572.
139. Yecies, LD, Wedner, HJ, Johnson, SM, Jakschik, BA and Parker, CW. Slow reacting substance (SRS) from ionophore A23187 stimulated peritoneal mast cells of the normal rat. I. Conditions of generation and initial characterization. *J Immunol* 1979; 122:2083.
140. Yecies, LD, Johnson, SM, Wedner, HJ and Parker, CW. Slow reacting substance (SRS) from ionophore A23187 stimulated peritoneal mast cells of the normal rat. II. Evidence for a precursor role of arachidonic acid and further purification. *J Immunol* 1979; 122:2090.
141. Atkinson, JP, Weiss, A, Ito, M, Kelly, J and Parker, CW. Effects of ascorbic acid and sodium ascorbate on cyclic nucleotide metabolism in human lymphocytes. *J Cyclic Nucleotide Res* 1979; 5:107.
142. Stenson, WF and Parker, CW. Metabolism of arachidonic acid in ionophore stimulated neutrophils. Esterification of an hydroxylated metabolite into phospholipids. *J Clin Invest* 1979; 64:1457.
143. Kennerly, DA, Secosan, CJ, Parker, CW and Sullivan, TJ. Modulation of stimulated phospholipid metabolism in mast cells by pharmacologic agents which increase cyclic 3', 5' adenosine monophosphate levels. *J Immunol* 1979; 123:1519.
144. Parker, CW, Jakschik, BA, Huber, MG and Falkenheim, SF. Characterization of slow reacting substance as a family of thiolipids derived from arachidonic acid. *Biochem Biophys Res Comm* 1979; 89:1186.
145. Wedner, HJ, Chan, BY, Parker, CS and Parker, CW. Cyclic nucleotide phosphodiesterase activity in human peripheral blood lymphocytes and monocytes. *J Immunol* 1969; 123:725.
146. Parker, CW, Kelly, JP, Falkenheim, SF and Huber, MG. Release of arachidonic acid from human lymphocytes in response to mitogenic lectins. *J Exp Med* 1979; 149: 1487.
147. Parker, CW. Role of cyclic nucleotides in regulating lymphocytes. *Ann NY Acad Sci* 1979; 332:255.
148. Philpott, GW, Grass, EH and Parker, CW. Affinity cytotoxicity with an alcohol dehydrogenase-antibody conjugate and allyl alcohol. *Ca Res* 1979; 39:2084.
149. Hempstead, BL, Parker, CW and Kulczycki, A Jr. Characterization of the IgE receptor isolated from human basophils. *J Immunol* 1979; 123:2283.
150. Kennerly, DA, Sullivan, TJ, Sylvester, P and Parker, CW. Diacylglycerol metabolism in mast cells: A potential role in membrane fusion and arachidonic acid release. *J Exp Med* 1979; 150:1039.

151. Kennerly, DA, Parker, CW and Sullivan, TJ. Use of diacylglycerol kinase to quantitative picomole levels of 1, 2diacylglycerol. *Analytic Biochem* 1979; 98:123.
152. Chaplin, DD, Wedner, HJ and Parker, CW. Protein phosphorylation in human peripheral blood lymphocytes. I. Subcellular distribution and partial characterization of adenosine 3', 5' monophosphate-dependent protein kinase and protein phosphorylation in human peripheral blood lymphocytes. *Biochem J* 1979; 182:525.
153. Chaplin, DD, Wedner, HJ and Parker, CW. Protein phosphorylation in human peripheral blood lymphocytes. II. Phosphorylation of endogenous plasma membrane and cytoplasmic proteins. *Biochem J* 1979; 182:537.
154. Stenson, WF and Parker, CW. 12-L-hydroxy 5,8,10,14-eicosatetraenoic acid, a chemotactic fatty acid, is incorporated into neutrophil phospholipids and triglyceride. *Prostaglandins*. 1979; 18:285.
155. Yecies, LD, Wedner, HJ and Parker, CW. Slow reacting substance (SRS) from ionophore A13187 stimulated human leukemic basophils. I. Evidence for a precursor role of arachidonic acid and initial purification. *J Immunol* 1979; 123:2814.
156. Yecies, LD, Parker, CW and Watanabe, S. Slow reacting substances from ionophore A23187-stimulated basophilic leukemia cells and peritoneal mast cells in the rat. I. Purification and comparison during sequential Sephadex LH-20 and thin layer chromatography. *Life Sciences* 1979; 25:1909.
157. Parker, CW, Huber, MM, Hoffman, MK and Falkenheim, SF. Characterization of the two major species of slow reacting substance from rat basophilic leukemia cells as glutathionyl thioethers of eicosatetraenoic acids oxygenated at the 5 position. Evidence that peroxy groups are present and important for spasmogenic activity. *Prostaglandins* 1979; 18:673.
158. Parker, CW. Modulation of lymphoid cell function and allergic responses. *Adv Cyclic Nucleotide Res* 1980; 12:181.
159. Falkenheim, SF, MacDonald, H, Huber, MM, Koch, D and Parker, CW. Effect of the 5-hydroperoxide of eicosatetraenoic acid and inhibitors of the lipoxygenase pathway on the formation of the slow reacting substance by rat basophilic leukemia cells. Direct evidence that slow reacting substance is a product of the lipoxygenase pathway. *J Immunol* 1980; 125:163.
160. Philpott, GW, Kulczycki, A Jr., Grass, EH and Parker, CW. Selective binding and cytotoxicity of rat basophilic leukemia cells (RBL-1) with immunoglobulin E-biotin and avidin-glucose oxidase conjugates. *J Immunol* 1980; 125:1201.
161. Parker, CW, Koch, DA, Huber, MM and Falkenheim, SF. Incorporation of radiolabel from [1-¹⁴C]5-hydroxy-eicosatetraenoic acid into slow reacting substance. *Biochem Biophys Res Comm* 1980; 94:1037.
162. Stenson, WF and Parker, CW. Monohydroxyeicosatetraenoic acids (HETEs) induce degranulation of human neutrophils. *J Immunol* 1980; 124:2100.
163. Stenson, WF and Parker, CW. Prostaglandins, macrophages and immunity (Invited Editorial). *J Immunol* 1980; 125:1.
164. Chaplin, DD, Wedner, HJ and Parker, CW. Protein phosphorylation in human peripheral blood lymphocytes. Mitogen-induced increases in protein phosphorylation in intact lymphocytes. *J Immunol* 1980; 124:2390.
165. Kohno, S and Parker, CW. Role of arachidonic acid in the biosynthesis of slow reacting substance of anaphylaxis (SRS-A) from sensitized guinea pig lung fragments. Evidence that SRS-A is very similar or identical structurally to non-immunologically induced forms of SRS. *J Immunol* 1980; 125:946.
166. Gordon, LK, Hamill, B and Parker, CW. The activation of blast transformation and DNA synthesis in human peripheral blood lymphocytes by wheat germ agglutinin. *J Immunol* 1980; 125:814.

167. Udey, MD, Chaplin, DD, Wedner, HJ and Parker, CW. Early activation events in lectin-stimulated human lymphocytes. Evidence that wheat germ agglutinin and mitogenic lectins cause similar early changes in lymphocyte metabolism. *J Immunol* 1980; 125: 1544.
168. Udey, MC and Parker, CW. Cell biology of lymphocyte activation. *Fed Proceed* 1980; 39:14, 3159.
169. Parker, CW, Fischman, CM and Wedner, HJ. Relationship of slow reacting substance biosynthesis to intracellular glutathione levels. *Proc Natl Acad Sci USA* 1980; 77:6870.
170. Parker, CW, Falkenheim, SF and Huber, MM. Sequential conversion of glutathionyl show reacting substance (SRS) to cysteinyl SRS in rat basophilic leukemia cells stimulated with A23187. *Prostaglandins* 1980; 20:863.
171. Parker, CW, Koch, DA, Huber, MM and Falkenheim, SF. Arylsulfatase inactivation of slow reacting substance. Evidence for proteolysis as a major mechanism when ordinary commercial preparations of the enzyme are used. *Prostaglandins* 1980; 20:887.
172. Parker, CW, Koch, D, Huber, MM and Falkenheim, SF. Formation of the cysteinyl form of slow reacting substance (Leukotriene E₄) in human plasma. *Biochem Biophys Res Comm* 1980; 97:1083.
173. Stenson, WF, Parker, CW and Sullivan, TJ. Augmentation of IgE-mediated release of histamine by hydroxyeicosatetraenoic acid and 12-hydroxyeicosatetraenoic acid. *Biochem Biophys Res Comm* 1980; 96:1045.
174. Sullivan, TJ, Wedner, HJ and Parker, CW. Desensitization of patients allergic to penicillin using oral penicillin. *J Allergy Clin Immunol* 1980; 65:195.
175. Parker, CW. Radioimmunoassay. *Ann Rev Pharmacol Toxicol* 1981; 21:113.
176. Udey, MC and Parker, CW. Membrane protein synthesis in mitogen stimulated human T lymphocytes. *J Immunol* 1981; 126:1106.
177. Hempstead, BL, Kulczycki, A Jr. and Parker, CW. Phosphorylation of the IgE receptor from ionophore A23187 stimulated intact rat mast cells. *Biochem Biophys Res Comm* 1981; 98:815.
178. Gordon, LK and Parker, CW. A microfluorometric assay for the measurement of *de novo* DNA synthesis in individual cells. *J Immunol* 1981; 127:1634.
179. Hempstead, BL, Parker, CW and Kulczycki, A Jr.. The cell surface receptor for immunoglobulin E. Effect of tunicamycin on molecular properties of receptor from RBL-1 cells. *J Biol Chem* 1981; 256: 10717.
180. Parker, CW. Autoantibodies and β adrenergic receptors. Editorial to accompany paper by D. M. Fraser, "Autonomic abnormalities and autoantibodies to β adrenergic receptors". *New Eng J Med* 1981; 305:1212.
181. Sullivan, TJ, Wedner, HJ, Shatz, GS, Yecies, LD and Parker, CW. Skin testing to detect penicillin allergy. *J Allergy Clin Med* 1981; 68:171.
182. Parker, CW. Hapten immunology and allergic reactions in man. Kroc Symposium on Drug Induced Lupus. *Arthritis and Rheumatism* 1981; 24:1024.
183. Udey, MD and Parker, CW. Effect of inhibitors of arachidonic acid metabolism on a γ -aminoisobutyric acid transport in human lymphocytes. *J Biochem Pharm* 1982; 31:337.
184. Parker, CW. Allergic reactions in man. *Pharm Rev* 1982; 34:104.
185. Parker, CW. Pharmacologic modulation of release of arachidonic acid from human mononuclear cells and lymphocytes. *J Immunol* 1982; 128:393.
186. Kreisle, RA and Parker, CW. Uptake of leukotriene B₄ by human polymorphonuclear leukocytes. *FASEB* 1982; 41:373.
187. Parker, CW, Huber, MM and Falkenheim, SF. Pharmacologic characterization of slow reacting substances. *Methods in Enzymology* 1982; 6:655.
188. Parker, CW, Falkenheim, SF and Huber, MM. Production and purification of slow reacting substance (SRS) from RBL-1 cells. *Methods in Enzymology* 1982; 86:416.
189. Udey, MD, Jendrisak, MD and Parker, CW. Properties of a surface antigen expressed on activated human thymus-derived lymphocytes. *J Immunol* 1982; 128:1870.

190. Sullivan, CM, Yecies, LR, Shatz, GS, Parker, CW and Wedner, HJ. Desensitization of patients allergic to penicillin using orally administered P lactin antibodies. *J Allergy Clin Immunol* 1982; 69:275.
191. Findlay, SR, Leary, TM, Parker, CW, Bloomquist, EI and Scheid, CR. Contractile events induced by LTD₄ and histamine in smooth muscle - a function of calcium utilization. *Amer Physiol Soc* 1982; C133.
192. Parker, CW and Aykent, S. Calcium stimulation of the 5-lipoxygenase from RBL-1 cells. *Biochem Biophys Res Comm* 1982; 109:1011.
193. Parker, CW, Schechtel, T, Falkenheim, S and Huber, MM. Induction of IgE receptors on human lymphocytes. *Immunol Comm* 1983; 12:1.
194. Kreisle, RA and Parker, CW. Specific binding of leukotriene B₄ to a receptor on human polymorphonuclear leukocytes. *J Exp Med* 1983; 157:628.
195. Wahl, RL, Philpott, GW and Parker, CW. Improved radio imaging with F(Ab')₂ fragments of monoclonal antibody. *J Nuclear Med* 1983; 24:316.
196. Wahl, RL, Parker, CW and Philpott, GW. Monoclonal antibody radioimmuno-detection of human derived colon carcinoma. *Invest Radiology* 1983; 18:58.
197. Milstone, D and Parker, CW. Purification of primate T cell growth factor to apparent homogeneity by reverse phase high pressure liquid chromatography: Evidence for two highly active molecularly distinct species. *Biochem Biophys Res Comm* 1983; 115:762.
198. Hempstead, BL, Parker, CW and Kulczycki, A Jr. Selective phosphorylation of the IgE receptor in antigen-stimulated rat mast cells. *Proc Natl Acad Sci USA* 1983; 80:3050.
199. Parker, CW. Lipoxygenase and leukotrienes. Kroc Foundation Symposium. *J Allergy Clin Immunol* 1984; 74:343.
200. Wahl, RL and Parker, CW. Monoclonal antibody radioimmuno-detection of transplant rejection. *Transplantation* 1985; 40:451.
201. Kreisle, RA, Parker, CW, Griffin, GL, Senior, RM and Stenson, WF. Studies of leukotriene B₄-specific binding and function in rat polymorphonuclear leukocytes: Absence of a chemotactic response. *J Immunol* 1985; 134:3356.
202. Kurosawa, M and Parker, CW. A phosphatidylinositol kinase in rat mast cell granules. *J Immunol* 1986; 136:616.
203. Parker, CW. Allergic activation of mast cells. *Jpn J Allergol* 1986; 35:305.
204. Milstone, DS and Parker, CW. The glycoprotein nature and biosynthetic relationship of two molecularly distinct species of primate T-cell growth factor. *Biochem J* 1986; 236:371.
205. Sternberg, EM, Trial, J and Parker, CW. Effect of serotonin on murine macrophages. 1. Suppression of Ia expression and its reversal by type 2 serotonin antagonists. *J Immunol* 1986; 137:276.
206. Kurosawa, M and Parker, CW. Characterization of calcium-activated phospholipid-dependent protein inase from rat serosal mast cells and RBL-1 cells. *Cell Immunol* 1986; 103:381.
207. Kurosawa, M and Parker, CW. Protein phosphorylation in rat mast cell granules. Cyclic AMP dependent phosphorylation of a 44K protein inside the granule. *Biochem Pharmacol* 1987; 36:131.
208. Panuska, J and Parker, CW. Radioiodination of proteins by reductive alkylation. *Anal Biochem* 1987; 160:192.
209. Parker, CW. Lipid mediators produced through the lipoxygenase pathway. *Ann Rev Immunol* 1987; 5:65.
210. Parker, CW. Various roles of lipids and lipid metabolizing enzymes in inflammatory processes and their possible implications for therapy. Transatlantic Airway Conference. *Am Rev Resp Dis* 1987; 135:S22.
211. Sternberg, EM, Wedner, HJ, Leung, MK and Parker, CW. Effect of serotonin (5-HT) and other monoamines on murine macrophages: Modulation of interferon-(induced phagocytosis. *J Immunol* 1987; 138:4360.

212. Parker, CW. 5-Lipoxygenase, leukotrienes, and regulation of inflammatory responses. *Drug Devel Res* 1987; 10:277.
213. Parker, CW. Biochemical changes taking place in mitogen-treated lymphocytes. *Meths. Enzymol.* 1987; 150:29.
214. Parker, CW. Fc epsilon receptors. *Meths. Enzymol* 1987; 150:512.
215. Panuska, J, Fukui, K and Parker, CW. Secreted proteins of human monocytes. Analysis by two-dimensional gel electrophoresis and effect of lipo polysaccharides. *Biochem J* 1988; 249:501.
216. Kurosawa, M and Parker, CW. Change in polyphosphoinositide metabolism during mediator release from stimulated rat mast cells. *Biochem Pharmacol* 1989; 38:431.
217. Parker, CW and Stenson, WF. Prostaglandins and leukotrienes. *Current Op in Immunol* 1989; 2:28.
218. Parker, CW. Section XI. Immunological procedures. Immunoassays. *Meths. Enzymol.* 1990; 82:700.
219. Parker, CW. Section XII. Additional techniques. Radiolabeling of proteins. *Meths. Enzymol* 1990; 182:721.
220. George, RJ and Parker, CW. Preliminary characterization of phosphotyrosine phosphatase activities in human peripheral blood lymphocytes: Identification of CD45 as a phosphotyrosine phosphatase. *J Cell Biochem* 1990; 42:71.
221. Parker, CW. Radiolabeling of proteins. *Methods in Enzymology* 1990; 182:721.
222. Parker, CW. Immunoassays. *Methods in Enzymology* 1990; 182:700.
223. Parker, CW. Environmental stress and immunity. Possible implications for IgE-mediated allergy. *Perspectives in Biology and Medicine* 1991; 34:197.
224. Parker, CW. Neutrophil mechanisms. [Review]. [25 refs]. *Am Rev of Resp Dis* 1991; 143:S59.
225. Parker, CW, Huber, MG, Godt, SM. Modulation of IL-4 production in murine spleen cells by prostaglandins. *Cellular Immunol* 1995; 160:278.

Clinico-Pathologic Conferences:

1. Parker, CW, Levitt, RG, Uitto, J, Bruce, RM, Roberts, R and Kissane, J. Mixed connective tissue diseases. *Amer J Med* 1978; 65:833.
2. Parker, CW, Sagel, S, Chaplin, H, Atkinson, J, Bruce, R and Kissane, J. Thrombocytopenia in a young girl. *Amer J Med* 1980; 68:595.
3. Parker, CW. Multiple infectious diseases and death in a homosexual male. *Amer J Med* 1973; 75:855.
4. Parker, CW. Serum and urine paraprotein and filling defects in the spleen. *Amer J Med* 1976; 60:531.
5. Parker, CW. Septic shock in a young splenectomized man. *Amer J Med* 1983; 74:129.
6. Parker, CW, Panuska, JR and Sternberg, E. Fever of unknown origin in a 71-year-old woman. *Amer J Med* 1985; 79:373.
7. Parker, CW. Sudden death in a 22-year-old woman with SLE. *Amer J Med* (Presented 12/85).
8. Parker, CW. 43-year-old homosexual male with Kaposi's sarcoma. *Amer J Med* (Presented 12/88).

(b) Other Invited Publications

1. Parker, CW. Antibody-hapten reactions as measured by a spectrofluorometric method. In: Conceptual Advances in Immunology and Oncology. Hoeber, New York, 1963, pp. 191-205.

2. Parker, CW. Penicilloyl-polylysines. Preparation, properties and analytical procedures, with special reference to their use in human skin testing. In: Methods in Medical Research. Yearbook Medical Publishers, 1964; 10:192.
3. Parker, CW. Chapter in Clinical Section on Drug Allergy. In: Immunological Diseases. Little, Brown and Co., Boston, Massachusetts, 1965, pp. 663-681.
4. Parker, CW. Allergic responses. In: Clinical Dermatology. (Eds.) DJ Demis, RG Crounse, R. Dobson and J. McGuire. Harper and Row, New York. 1966; 3:1.
5. Parker, CW. Spectrofluorometric methods. In: Handbook of Experimental. Blackwells Scientific Publications, Oxford, 1964, pp. 423-462.
6. Parker, CW. Conjugating penicillin and its derivatives. In: Methods in Immunology and Immunochemistry. Academic Press, New York, 1967, pp. 133-143.
7. Parker, CW and Halloran, MJ. The production of antibodies to mononucleotides, oligonucleotides and DNA. In: Nucleic Acids in Immunology. Springer-Verlag, New York, 1968, pp. 18-31.
8. Parker, CW and Vavra, JD. Immunosuppression. In: Progress in Hematology. Vol. VI, Grune and Stratton, New York, 1969, pp. 1-81.
9. Parker, CW. Urticaria, contact eczema and iso- and autoimmune phenomena. Associated with skin in experimental systems and in non-bullous human diseases. In: Textbook of Immunopathology. Vol. I and II, Grune and Stratton, New York, 1969.
10. Parker, CW. Immediate skin reactions in penicillin hypersensitivity. In: Penicillin Allergy: Clinical and Immunologic Aspects. (Eds) GT Stewart and JP McGovern. Charles C. Thomas, Springfield, Illinois, 1970.
11. Steiner, AL, Parker, CW and Kipnis, DM. The measurement of cyclic nucleotides by radioimmunoassay. In: Advances in Biochemical Psychopharmacology. Raven, Press, New York, 1970, p. 89.
12. Parker, CW. Chapter 14. In: Dermatology in General Medicine. McGraw Hill, New York, 1971, p. 699.
13. Parker, CW. Radioimmunoassays. In: Progress in Clinical Pathology. Vol. IV, Grune and Stratton, New York, 1971.
14. Parker, CW. The nature of immunological responses and antigen-antibody interactions. In: Principles of Competitive Protein-Binding Assays. (Eds). WD Odell and WH Daughaday. J.B. Lippencott Co., Philadelphia, 1971, pp. 25-48.
15. Parker, CW. Section in Immunologic Intervention. (Eds). JW Uhr and M. Landy. Academic Press, New York, 1971.
16. Parker, CW. The role of prostaglandins in the immune response. In: Prostaglandins in Cellular Biology. (Eds). PW Ramwell and BB Pharriss. Plenum Press, New York, 1972, p. 173.
17. Parker, CW. Practical aspects of diagnosis and treatment of patients who are hypersensitive to drugs. In: International Encyclopedia of Pharmacology and Therapeutics. (Eds). M. Samter and CW Parker. Pergamon Press, New York, 1972.
18. Parker, CW. Cyclic AMP. In: Inflammation: Mechanism and Controls. (Eds) IH Lepow and PA Ward. Academic Press, New York, 1972, p. 239.
19. Parker, CW. Allergic drug responses. mechanisms and unsolved problems. In: Critical Reviews in Toxicology. Vol. I, CRC Press, Cleveland, Ohio, 1972, pp. 261-281.
20. Parker, CW. The paradox of prostaglandins in asthma. In: Perspectives on the Prostaglandins. (Ed). JB Lee. Medcom Press, New York, 1972, p. 33.
21. Jaffee, BM, Philpott, GW, Hamprecht, B and Parker, CW. Prostaglandin production by cells *in vitro*. In: Advances in Biosciences. Vol. 9, Pergamon Press, New York, 1972.
22. Aach, RD, Hacker, E and Parker, CW. Radioimmunoassay for hepatitis-associated antigen (HBAG) and antibodies to HBAG. In: Proceedings of the Symposium on Viral Hepatitis and Blood Transfusion. Grune and Stratton, New York, 1972.

23. Jaffe, BM, Parker, CW and Philpott, GW. Prostaglandin release by human cells *in vitro*. In: Prostaglandins in Cellular Biology. (Eds). PW Ramwell and BB Pharriss. Plenum Press, New York, 1972, pp. 207-226.
24. Parker, CW. Mechanisms of penicillin allergy. In: Pathobiologic Annual. Appleton-Century-Crofts, New York, 1972, pp. 405-417.
25. Parker, CW. Spectrofluorometric methods. In: Handbook of Experimental Immunology. (Ed) DM Weir. Blackwell Scientific Publications, Oxford, 1973, pp. 423-461.
26. Lafferty, JJ, Kannegiesser, H, Lee, JB and Parker, CW. Metabolic mechanisms of action of the renal prostaglandin. In: Proceedings of a Conference of Prostaglandins. Vienna, 1973.
27. Parker, CW, Bower, FJ, Aach, RD and Philpott, GW. The immunologic therapy of cancer. In: Pharmacology and the Future of Man. Vol. 5, S. Karger, Basel, 1973, pp. 411-419.
28. Jaffe, BM and Parker, CW. Extraction of PGE from human serum for radioimmunoassay. In: Prostaglandins in Fertility Control. Karolinska Institute, Stockholm, 1973, p. 69.
29. Parker, CW. Adrenergic responsiveness in asthma. In: Asthma: Physiology, Immunopharmacology and Treatment. (Eds) LM Lichtenstein and KF Austen. Academic Press, New York, 1973, pp. 185-206.
30. Parker, CW. Urticaria and contact eczema. In: Textbook of Immunopathology. (Revision) Grune and Stratton, New York, 1973.
31. Shearer, WT and Parker, CW. Heterogeneity of IgG determined by fluorescence. In: Biochemical Fluorescence: Concepts. (Eds) RF Chen and H. Edelhoch. Marcel Dekker, New York, 1976, pp. 811-843.
32. Parker, CW, Sullivan, TJ and Wedner, HJ. Cyclic AMP and the immune response. In: Advances in Cyclic Nucleotide Research. (Eds) GA Robison and P. Greengard. Raven Press, New York, 1974, pp. 1-79.
33. Parker, CW. Complexities in the study of the role of cyclic nucleotides in lymphocyte responses to mitogens. In: Cyclic AMP, Cell Growth and the Immune Response. (Eds) W. Braun, LM Lichtenstein and CW Parker. Springer-Verlag, New York, 1974, pp. 35-44.
34. Parker, CW. Immunological methods for the detection of drugs; their application in the detection of digitoxin, digoxin and morphine. In: The New Poisoned Patient: The Role of the Laboratory. Ciba Foundation Symposium, Elsevier, Amsterdam, 1974, pp. 201-217.
35. Jaffe, BM and Parker, CW. Radioimmunoassay for prostaglandins. In: Radioassay in Clinical Medicine. (Eds) TW Newton and RM Donati. Charles C. Thomas, Springfield, 1974, pp. 33-48.
36. Parker, CW, Snider, DE and Wedner, HJ. The role of cyclic nucleotides in lymphocyte activation. In: Progress in Immunology II. (Eds) L. Brent and J. Holborrow. North-Holland Publishing Co., New York, 1974, pp. 85-94.
37. Wedner, HJ, Bloom, FE and Parker, CW. The role of cyclic nucleotides in lymphocyte proliferation. In: Immune Recognition. (Ed) AS Rosenthal. Academic Press, New York, 1975, pp. 337-357.
38. Parker, CW. Possible mechanisms of lymphocyte activation. In: Immune Recognition. (Ed) AS Rosenthal. Academic Press, New York, 1975, p. 331.
39. Wedner, HJ and Parker, CW. Lymphocyte activation. In: Progress in Allergy. Vol. 20. Karger, Basel, 1976, pp. 195-300.
40. Parker, CW. Cyclic nucleotide metabolism of reticuloendothelial cells. In: Advances in Experimental Medicine and Biology. Plenum Press, New York, 1976, pp. 131-138.
41. Parker, CW. Radioimmunoassay of Biologically Active Compounds. Prentice-Hall, New York, 1976.
42. Atkinson, JP and Parker, CW. Autoimmunity associated with skin in experimental systems and in non-bullous human disease. In: Textbook of Immunopathology. (Eds) PA Miescher and HJ Muller Eberhard. Grune and Stratton, New York, 1976, pp. 433-441.

43. Green, WC, Wedner, HJ and Parker, CW. Calcium Ionophore A23187 and lymphocyte activation. In: Leukocyte Membrane Determinants Regulating Immune Reactivity. (Eds) VP Eijssvoegel, D. Roos and WP Zijlemaker. Academic Press, New York, 1976, p. 129.
44. Parker, CW, Green, WC. Cyclic nucleotides in the immune response. In: Cyclic Nucleotides: Mechanisms of Action. (Eds) H. Cramer and J. Schultz. John Wiley and Sons, New York, 1976, pp. 161-198.
45. Parker, CW. Urticaria. In: Textbook of Immunopathology. (Eds) PA Miescher and HJ Muller Eberhard. Grune and Stratton, New York, 1976, pp. 369-376.
46. Parker, CW. Contact eczema. In: Textbook of Immunopathology. (Eds) PA Miescher and HJ Muller Eberhard. Grune and Stratton, New York, 1976, pp. 433-441.
47. Atkinson, JP and Parker, CW. Autoimmunity associated with skin in experimental systems and in nonbullous human diseases. In: Textbook of Immunopathology. (Eds) PA Miescher and HJ Muller Eberhard. Grune and Stratton, New York, 1976, pp. 925-930.
48. Parker, CW. New directions in prostaglandin research and their possible implications for immunologic and nonimmunologic inflammation. In: Monographs in Allergy. S. Karger, New York, 1977, pp. 150-154.
49. Parker, CW. Problems in identification for responsible antigenic determinants in drug allergy. In: Drug Design and Adverse Reactions. (Eds) H. Bundgaard, P. Juul and H. Kofod. Academic Press, New York, 1977, pp. 153-160.
50. Parker, CW. Spectrofluorometric methods. In: Handbook of Experimental Immunology. Blackwells Scientific Publication, Oxford, England, 1977.
51. Parker, CW. Aspirin sensitive asthma. In: Asthma: Physiology, Immunopharmacology and Treatment. (Eds) LM Lichtenstein, KF Austen and AS Simon. Academic Press, New York, 1977, pp. 301-313.
52. Wedner, HJ, Chaplin, DD and Parker, CW. Evidence for an early sulfhydryl reagent sensitive step during lymphocyte activation. In: Proceedings of the 11th Leukocyte Culture Conference. Academic Press, New York, 1977, p. 456.
53. Parker, CW. Potential usefulness of monoclonal culture techniques. In: Antibodies in Human Diagnosis and Therapy. (Eds) E. Haber and RM Krause. Raven Press, New York, 1977, p. 237.
54. Snider, DE and Parker, CW. Prostaglandins. In: Allergy: Principles and Practice. (Eds) E. Middleton, E. Ellis and C. Reed. C. V. Mosby Co., St. Louis, MO, 1978, pp. 531-547.
55. Parker, CW. Drug allergy. In: Burger's Medicinal Chemistry. (Ed) ME Wolff. John Wiley and Sons, New York, 1979, pp. 419-454.
56. Parker, CW. Autosensitization dermatitis. In: Dermatology in General Medicine. (Eds) T. B. Fitzpatrick, WH Clark, Jr., AZ Eisen, KF Austin, IM Freedberg and K Wolff. McGraw-Hill, Inc, New York, 1979, pp. 530-531.
57. Parker, CW. The role of intracellular mediators. In: Biology of the Lymphokines. (Eds) E. Pick, J. Oppenheim and S. Cohen. Academic Press, New York, 1979, pp. 541-583.
58. Chaplin, DD, Wedner, HJ and Parker, CW. Protein phosphorylation and lymphocyte activation. In: Proceedings of the International Symposium on the Biological Basis for Immunodeficiency. Toronto, October, 1978. Raven Press, New York, 1980, pp. 269-281.
59. Roberts, R and Parker, CW. Radioimmunoassay for human creatine kinase isoenzymes. In: Methods in Enzymology: Immunochemical Techniques. (Eds) H. Van Vunakis and JJ Langone. Academic Press, New York, 1980.
60. Parker, CW (Ed) Clinical Immunology, Two Volumes. W.B. Saunders Co., Philadelphia, 1980, pp. 1-1438.
61. Parker, CW. SRS-A of rat basophil leukemia and rat mast cells. In: Proceedings of the Fourth International Symposium on the Biochemistry of the Acute Allergic Reaction. Alan R. Liss, Inc., New York, 1980, pp. 23-36.

62. Parker, CW. Role of mast cells and basophils in human allergy. In: Proceedings of the National Eye Institute Workshop in Infection, Inflammation and Allergy. Information Retrieval, Inc., New York, 1980.
63. Parker, CW. Pulmonary effects of inhibitors. In: Prostaglandins and related Lipids. (Ed) P. Ramwell. Alan R. Liss, Inc., New York, 1980, pp. 31-43.
64. Parker, CW. Arachidonic acid and related fatty acids in leukocyte modulation. In: Proceedings of the First International Conference of Immunopharmacology. (Eds) Hadden, et al. Oxford, Pergamon Press, Brighton, England, 1981.
65. Parker, CW. Incorporation of radio-labeled precursor into SRS-A. In: SRS-S and Leukotrienes. (Ed) PJ Piper. John Wiley and Sons, Ltd., New York, 1981, pp. 131-150.
66. Parker, CW. Arachidonic acid metabolism in activated lymphocytes. In: Mechanisms of Lymphocyte Activation. (Eds) K. Resch and H. Kirchner. Elsevier/North Holland Biomedical Press, New York, 1981, pp. 47-57.
67. Stenson, WF and Parker, CW. The immune system. In: Comprehensive Endocrinology. (Ed) JB Lee. Elsevier/North Holland, New York, 1982, pp. 39-89.
68. Stenson, WF and Parker, CW. Prostaglandins. In: Immunopharmacology. Centre Hospitalier Universitaire. Elsevier/North-Holland Biomedical Press B.V., 1982.
69. Parker, CW. Leukotrienes. Their metabolism, structure and role in allergic responses. In: Leukotrienes and Other Lipoxygenase Products. (Eds) B. Samuelson and R. Paoletti. Raven Press, New York, 1982.
70. Parker, CW. The chemical nature of slow-reacting substances. In: Advances in Inflammation Research. (Ed) G. Weissmann. Raven Press, New York, 1982, Vol. 4, pp. 1-24.
71. Parker, CW. Arachidonate metabolites and immunity. In: Prostaglandins and Related Lipids. Vol. 2 For: Prostaglandins and Cancer: First International Conference. (Eds) TJ Powles, RS Bockman, KV Honn and P. Ramwell. Alan R. Liss, Inc., New York, 1982, pp. 595-607.
72. Stenson, WF, Snider, DE and Parker, CW. Prostaglandins and other arachidonate metabolites. In: Allergy: Principle and Practice. (Ed) E. Middleton, C.W. Mosby Co., St. Louis, MO 1983.
73. Parker, CW. Immunopharmacology of slow reacting substances. In: Immunopharmacology of the Lung. (Ed) HH Newball. Marcel Dekker, Inc., New York, 1983, pp. 25-53.
74. Stenson, WF and Parker, CW. Metabolites of arachidonic acid. In: Clinical Reviews in Allergy. (Ed) S. Wasserman. Elsevier Science Publishing Co., New York, 1983, pp. 369-384.
75. Parker, CW. Major drugs acting on the central nervous system. In: Handbook of Experimental Pharmacology. (Ed) A. deWeck. Springer-Verlag, New York, 1983, pp. 243-258.
76. Parker, CW. Allergic mediators. In: Allergy: Theory and Practice. (Eds) HJ Wedner and PE Korenblat. Grune and Stratton, New York, 1984, pp. 25-39.
77. Parker, CW. Mediators: Release and function. In: Fundamental Immunology. (Ed) We Paul. Raven Press, New York, 1984, pp. 697-747.
78. Stenson, WF and Parker, CW. Leukotrienes. In: Advances in Internal Medicine. (Eds) GH Stollerman, WJ Harrington, JT Lamont, JJ Leonard and MD Siperstein. Yearbook Medical Publishers, Inc., 1984, pp. 175-199.
79. Parker, CW. Intracellular activation in mast cells and lymphocytes. In: Progress in Immunology V. Academic Press, Japan, 1984, pp. 327-337.
80. Parker, CW. The lipoxygenase in leukocytes and other mammalian cells. In: The Chemistry and Biology of the Leukotrienes and Related Substances. (Eds) LW Chakrin and DM Bailey. Academic Press, New York, 1984, pp. 125-137.
81. Sternberg, E and Parker, CW. Pharmacologic aspects of lymphocyte regulation. In: The Lymphocyte: Structure and Function. (Ed) JJ Marchalonis. Marcel Dekker, Inc., New York, 1985, pp. 1-54.
82. Parker, CW. 5-Lipoxygenases. In: Advances in Prostaglandin, Thromboxane and Leukotriene Research. Kyoto Conference of Prostaglandins. Vol. 15, Chapter 45. (Eds) O. Hayaishi and S. Yamamoto. Raven Press, New York, 1985, pp. 167-168.

83. Parker, CW. Lipid mediators and inflammation. In: New Trends in Allergy II. (Eds) J. Ring and G. Burg. Springer-Verlag, Berlin, 1986, pp. 78-89.
84. Parker, CW. Leukotrienes and prostaglandins in the immune system. In: Advances in Prostaglandin, Thromboxane and Leukotriene Res. Vol. 16. (Eds) U. Zor, Z. Naor and F. Kohen. Raven Press, New York, 1986, pp. 113-114.
85. Parker, CW. Radioimmunoassay of cyclic nucleotide. In: Handbook of Experimental Pharmacology. Vol. 82. Radioimmunoassay in Basic and Clinical Pharmacology. (Eds) C. Patrono and B. A. Peskar. Springer-Verlag, Heidelberg, 1987, pp. 501-515.
86. Parker, CW. Immunological mechanisms. In: Highlights in Asthmology. (Eds) FB Michel and J. Bousquet. Springer-Verlag, Berlin, 1987, pp. 117-119.

THE PREPARATION OF NUCLEOTIDE-PROTEIN CONJUGATES: CARBODIIMIDES AS COUPLING AGENTS

MARK J. HALLORAN¹ AND CHARLES W. PARKER²

*From the Immunology Division, Department of Medicine, Washington University School of Medicine,
St. Louis, Missouri*

Received for publication August 2, 1965

For some time it has been apparent that antibodies might be useful in the study of the fine structure of RNA and DNA. A major stumbling block has been the unavailability of a method which would render polynucleotides antigenic yet largely preserve their structural integrity. In approaching the problem of covalently conjugating polynucleotides to proteins as a means of stimulating antibody formation, we sought a procedure which would employ terminal nucleotide PO₄ or OH groups for coupling. Two ways in which the terminal PO₄ groups might be coupled covalently to protein would involve formation of a phosphodiester bond with protein seryl and threonyl residues or an N-P bond with protein ε-amino groups (Fig. 1, reactions 1 and 2 respectively). On the other hand, the OH group of a terminal sugar residue could react with protein carboxyl groups forming an ester (Fig. 1, reaction 3). Among possible coupling agents, the water soluble carbodiimides seemed especially attractive because they are known to promote the formation of all three types of bonds (1). Evidence will be presented in this communication which indicates that nucleotides do couple to proteins and polylysine under very mild conditions, in the presence of carbodiimides. Investigation strongly implicates formation of N-P bonds as the principle type of linkage. In the accompanying article it will be shown that conjugates of proteins with mononucleotides, oligonucleotides and DNA elicit the formation of antibodies with nucleotide specificity (2). A brief resume of this work has been reported earlier (3).

¹ Postdoctoral Fellow of the United States Public Health Service, Grant 2 T1-A1-219.

² Recipient of a Research Career Development Award from the United States Public Health Service.

MATERIAL AND METHODS

1-Ethyl-3-diisopropylaminocarbodiimide-HCl (EDC)¹ was obtained from the Ott Chemical Company, Muskegon, Michigan. 1-3-Dicyclohexylcarbodiimide (DCC) and 1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl)-carbodiimide metho-*p*-toluenesulfonate (CMC) were obtained from the Aldrich Chemical Company, Milwaukee, Wisconsin. *N*-ethyl-5-phenylisoxazolium-3'-sulfonate, various mononucleotides, purified proteins, snake venom phosphodiesterase and calf intestine phosphomonoesterase, calf thymus and salmon sperm DNA were obtained from the Sigma Chemical Company, St. Louis, Missouri. Puromycin was a gift of Dr. Lillian Recant, Washington University School of Medicine. Polylysine-HBr was a product of the Pilot Chemical Company, Watertown, Massachusetts (MW 70,000-80,000).

3'-O-Acetyl thymidylic acid was synthesized according to the procedure of Gilham and Khorana (4). Tetrathymidylic acid was prepared and purified by the method of Khorana and Vizsolyi (5). The *N*-butylamine phosphoroamidate of adenylic acid was prepared as described in reference (6).

Coupling of mono- and oligonucleotides to proteins. The following will serve as an example of the procedure used in the coupling of mono-

¹ The following abbreviations are used throughout this article: 1-ethyl-3-diisopropylaminocarbodiimide-HCl, EDC; 1-3-dicyclohexylcarbodiimide, DCC; 1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl)-carbodiimide metho-*p*-toluenesulfonate, CMC; bovine γ-globulin, B₇G; *N*-ethyl-5-phenylisoxazolium-3'-sulfonate, reagent K; human serum albumin, HSA; thymidylic acid, T5'-PO₄; 3'-O-acetyl thymidylic acid, 3'-O-Ac-T5-PO₄; tetrathymidylic acid, (T5)₄; T5'-PO₄-DCC-HSA and T5'-PO₄-CMC-HSA are the protein conjugates of T5'-PO₄ with HSA in the presence of the respective coupling agent.

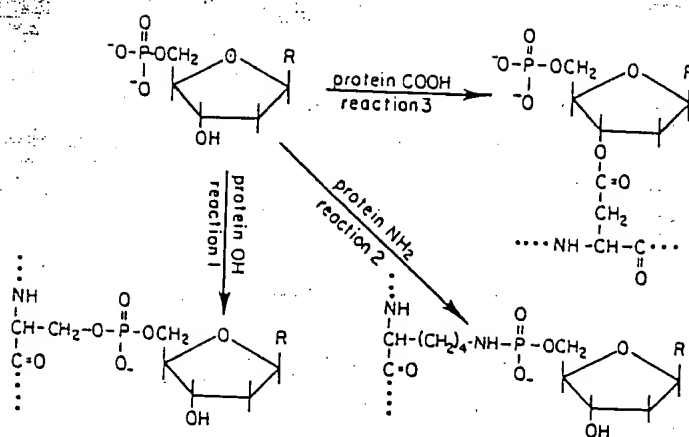


Figure 1. Possible reactions of nucleotides with proteins in the presence of carbodiimides. Very unstable products such as acyl phosphates are not shown.

nucleotides to protein: Human serum albumin (HSA), 25 mg, and 65 mg of thymidylic acid (T5'-PO₄) were dissolved in 0.5 ml H₂O. The pH was adjusted to 7.5 and 65 mg EDC were added. The reaction mixture was incubated for 24 hr at room temperature in the dark. The clear solution was dialyzed at 4°C against 0.01 M Tris chloride, pH 7.6, to a constant 267/230 mμ absorbency and phosphorus content (7). Protein concentration was measured by dry weight (8) and the Lowry technique (9).

The same general procedure was used in the coupling reactions involving adenylic acid, Puro-mycin, adenine, adenosine, thymidine and 3'-O-acetyl thymidylic acid to both bovine γ-globulin (BγG) and human serum albumin (HSA).

Coupling with another water soluble carbodi-imide, CMC, was carried out as described for EDC.

Seventy-five milligrams of dicyclohexylcarbodi-imide (DCC) was incubated for 10 min with 60 mg 3'-O-acetylthymidine 5'-PO₄ (3'-O-Ac-T5'-PO₄) in 1 ml of dry pyridine. The pyridine solution was then added dropwise with rapid stirring to a solution of 25 mg HSA in H₂O at pH 7.5. After 12 hr the reaction mixture was purified by dialysis as described above.

Attempts to couple T5 to proteins using Woodward's reagent K and tosyl chloride (10) were carried out under the conditions described above (e.g., in aqueous solution with and without preliminary incubation of the mononucleotide with the activating agent in dry pyridine).

Twenty-seven milligrams of tetrathymidylic

acid (T5), were reacted with 4.2 mg HSA and 13 mg EDC in 0.25 ml H₂O at pH 7.5. The product was dialyzed for 1 week at room temperature and 1 week at 4°C. During the final week of dialysis the absorbency of the protein (T5), solution at 267 mμ remained constant.

Coupling of DNA to protein. Highly polymerized salmon sperm DNA was denatured by boiling a 5 mg/ml solution for 10 min and then plunging the solution into a water bath containing crushed ice. Two milliliters of the denatured DNA solution were added to 20 mg of BγG dissolved in 1.5 ml of 0.1 N NaCl. The pH was adjusted to 7.5 and 20 mg EDC were added. The reaction mixture was allowed to incubate for 24 hr at room temperature in the dark and purified by dialysis against 0.01 M Tris-buffered saline. A small amount of precipitate which formed during the incubation was removed by centrifugation at 10,000 rpm for 1 hr. The material was analyzed by electrophoresis employing cellulose acetate and urea starch gel with varying pH conditions. Electrophoresis in the absence of urea was performed on cellulose acetate in 0.05 M barbital, pH 8.6, and in 0.05 M carbonate, pH 10, for 3 hr at room temperature, at 0.4 ma/cm strip. Electrophoresis also was carried out in starch gel containing 7 M urea, 0.05 M formate, pH 3.4, at 125 v for 8 hr. Protein and DNA were localized by Amidoshwartz and Feulgen stains respectively. Controls included the protein alone, EDC-treated protein alone, DNA alone, and mixtures of DNA with untreated and EDC treated protein. The amounts of DNA and protein loaded on the strips in the

TABLE I

Extent of coupling of nucleosides and nucleotides to proteins and polyaminoacids

Macromolecule	Nucleotide, Nucleoside or Base	Coupling Agent	Degree of Substitution per Molecule of Carrier ^a	Reactive Groups per Molecule of Carrier
HSA	T5' PO ₄	EDC	23	
HSA ^b	T5' PO ₄	DCC	16	
HSA	T5' PO ₄	CMC	13	
HSA	Adenine	EDC	<1	
HSA	Thymidine	EDC	3	
HSA	Adenosine	EDC	5	
HSA	T5' PO ₄	Tosyl chloride	<0.5	
HSA	T5' PO ₄	Reagent K	<0.5	
B γ G	T5' PO ₄	EDC	28	
B γ G	T5' PO ₄	CMC	17	
Poly-400-lysine ^c	T5' PO ₄	EDC	100	400
Poly-400-lysine	3' O-Ac-T5' PO ₄	EDC	100	400
Hydroxyethyl-poly-400-lysine	T5' PO ₄	EDC	<2	>400
Poly-600-glutamate	T5' PO ₄	EDC	2	600

^a Conjugates were prepared, purified, and analyzed as described in the text.^b A mixture of DCC and T5'-PO₄ in dry pyridine was added dropwise to stirred protein solution (see Material and Methods).^c With minor changes in the reaction conditions, i.e., lowering total volume of the reaction mixture, conjugation in the ratio of 200 T5'-PO₄ groups per 400 lysyl residues could be obtained.

controls were comparable to the amounts present in the conjugates. amine treatment of conjugates was carried out as described in reference (14).

Coupling of nucleotides to polyamino acids. Ten milligrams of polylysine-HBr were dissolved in 0.25 ml H₂O and 35 mg of T5'-PO₄ were added. The pH was adjusted to 7.5 and 35 mg of EDC were added. The preparation was incubated for 24 hr at room temperature in the dark and then dialyzed as described above. In a similar fashion the reaction of T5'-PO₄ and EDC with polytyrosine and polyglutamic acids was evaluated.

To evaluate further the possible reaction of T5'-PO₄ with hydroxyl groups, hydroxylation of a high molecular weight polylysine with ethylene oxide was carried out.⁴ The polylysine was incubated at pH 8 to 9 with a 100-fold molar excess of ethylene oxide (with respect to polymer NH₂) for 12 hr and purified by dialysis. The ninhydrin reaction of the product was negative, indicating quantitative hydroxyethylation of amino groups. The hydroxyethyl-polylysine was then reacted with T5'-PO₄ at pH 7.5 in the presence of EDC.

Enzymatic digestions of conjugates with snake venom phosphodiesterase and calf intestine phosphomonoesterase were carried out as described in references (12) and (13). Hydroxyl-

⁴ For a description of the reaction of ethylene oxide with proteins, see reference (11).

RESULTS

Preliminary experiments conducted with thymidylic acid and human serum albumin in aqueous solution, in the presence of the water soluble carbodiimide 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), indicated that conjugation of the mononucleotide with protein had taken place. At neutral pH and room temperature a soluble product which contained 23 thymidylate residues/molecule was obtained (Table I). The absorption spectrum of the product as compared with those of the unsubstituted protein and of the protein reacted with EDC alone is shown in Figure 2. The difference spectrum corresponded very closely to that of free thymidylic acid in the 250 to 280 mμ region. The number of thymidylate residues on the protein as estimated by the ultraviolet spectrum corresponded closely to the value obtained by quantitative phosphorus analysis.

Subsequent studies indicated that the conjugation reaction could be extended to other mononucleotides and other proteins using several different carbodiimides (Table I). Even the water insoluble carbodiimide, DCC, could act

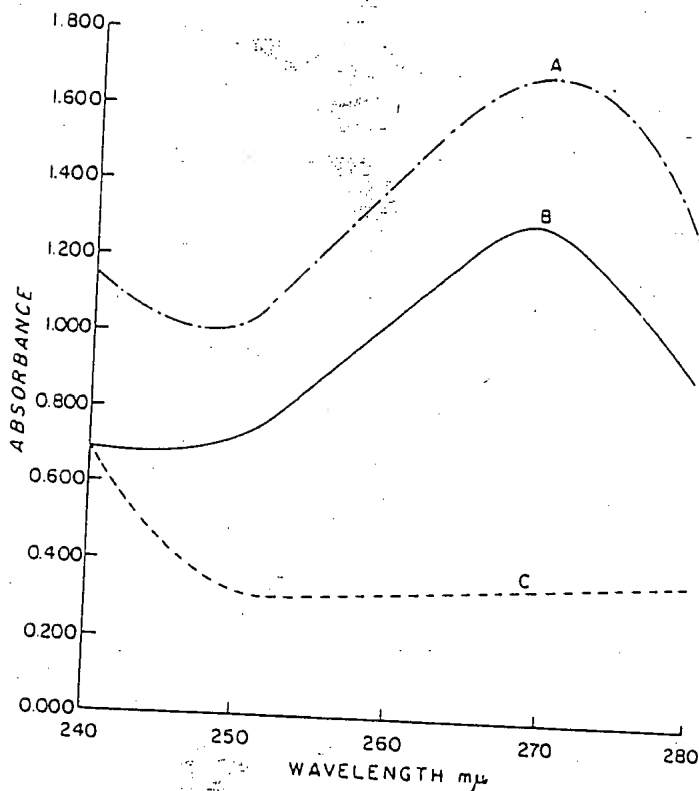


Figure 2. Absorption spectra of HSA and EDC-treated HSA (Curve C), and T5'-PO₄-EDC-HSA (curve A), each at concentrations of 0.7 mg/ml. The third curve (B) is a difference spectrum in which the protein contribution to the absorbance of T5'-PO₄-EDC-HSA has been subtracted.

as a coupling agent provided the nucleotide was incubated with DCC in dry pyridine before addition to the protein in aqueous solution (Table I). By contrast, Woodward's reagent K and tosyl chloride, two agents known to promote phosphodiester bond formation under anhydrous conditions, did not result in significant coupling.

In investigating the mechanism of the carbodiimide-induced conjugation of the nucleotide with protein, any substantial participation by the 3' OH group in an ester bond (Fig. 1, reaction 3) could be readily excluded: *a*) 3'-O-acetyl thymidylic acid, which lacks a reactive group at the 3' position, coupled as readily with protein and polylysine as thymidylic acid (Table I); *b*) when the conjugate of thymidylic acid with human serum albumin was subjected to alkaline hydrolysis at 37° in 0.1 M NaOH for various periods of time up to 1 hr and treated with hydroxylamine under conditions which cleave ester bonds (14), 85% or more of the phosphorous remained bound to protein; *c*)

thymidylic acid coupled very sluggishly with polyglutamic acid under the usual reaction conditions (Table I); *d*) while calf intestine phosphomonoesterase cleaved a portion of the phosphorous from protein (see below), thymidine also was removed. This indicated that cleavage was not taking place at a free 5'-PO₄ group.

In a similar manner it could be estimated that relatively few if any of the thymidylate-protein bonds were of the phosphodiester type (Fig. 1, reaction 1): *a*) thymidylate residues were not cleaved to a significant degree from thymidylate-HSA conjugates by snake venom phosphodiesterase; *b*) under the coupling conditions used for proteins, thymidylate acid failed to react appreciably with the hydroxyethylated derivative of polylysine (see Materials and Methods).

The results described above suggested that neither ester nor phosphodiester bonds could account for the majority of the protein-bound thymidylate, and focused attention on the possibility of an N-P bond as the predominant means

of combination (Fig. 1, reaction 2). In accord with this possibility it could be demonstrated that thymidylate readily reacted with polylysine producing a product with as many as 50% of ϵ -amino groups of polylysine substituted with thymidylate.⁵ Further evidence that N—P bonds can be formed in aqueous solution was obtained by paper chromatography of reaction mixtures containing butylamine, thymidylate acid and various carbodiimides.

On treatment of T5'-PO₄-HSA conjugates with calf intestine phosphomonoesterase at pH 9.5, thymidine and PO₄ were liberated from the protein, as judged by changes in phosphorous content and the ultraviolet spectrum after dialysis (Table II). Since the enzyme had not been subjected to rigorous purification, the presence of phosphoamidase activity could well account for this result. In accord with this possibility it was found that phosphoamide bonds on T5'-PO₄-polylysine and the *N*-butylamine phosphoamide of adenylate were cleaved by the enzyme.

While it was evident that mononucleotides could be coupled to proteins by means of carbodiimides, the applicability of the method to high molecular weight polymers remained to be established. Studies with a synthetically prepared tetramer of thymidylate (T5)₄ were encouraging; under the usual coupling conditions a conjugate of (T5)₄ with HSA was obtained which contained at least 50 (T5)₄ residues per molecule of protein. The method also appeared to be applicable to much larger units. Denatured salmon serum DNA (molecular weight 10 million) was reacted with B7G in the presence of EDC. The soluble product obtained was subjected to electrophoresis under a variety of conditions including 7 M urea in starch gel at pH 3.9. No condition was found which led to dissociation of the DNA and protein moieties. By contrast, with mock conjugates between native or EDC-treated protein and

⁵ Thymidylate acid also is capable of reacting with carbodiimides which contain secondary and tertiary amino groups. This reaction may assume some importance in protein conjugations using EDC or CMC. Judging from the results of Khorana and his colleagues with various amines, EDC should be capable of reacting directly with protein forming a 1-ethyl-3-diisopropylamino-guanidine substituent on the alkyl side chain of lysyl residues (1). It is possible that a portion of the thymidylate groups become bound to protein by coupling to ethyldiisopropylamino-guanidine.

TABLE II

Digestion of HSA-T5' PO₄-EDC and poly-L-400-T5' PO₄-EDC with phosphomonoesterase (calf intestine)^a

Enzyme		μ g P/mg Polylysine or Protein	O.D. at 267 m μ /mg Polylysine or Protein ^b
Poly-L-400-T5' PO ₄ -EDC	0	120	18.150
Poly-L-400-T5' PO ₄ -EDC	+	40	6.400
HSA-T5' PO ₄ -EDC	0	10	2.400
HSA-T5' PO ₄ -EDC	+	6.5	.960

^a Digestion was carried out on duplicate samples as described in Reference (13) followed by prolonged dialysis against 0.15 M saline, 0.001 N Tris chloride, pH 7.5. Control undigested samples were handled identically with the omission of enzyme.

^b Corrected for protein contribution at 267 m μ .

DNA, the protein and DNA could be readily separated. We would infer from these results that a stable linkage was formed between protein and DNA molecules in the presence of EDC.

Investigation of the reaction of adenosine and adenine with protein indicated that the 6-amino group of the base reacts very sluggishly, if at all, with proteins under the usual coupling conditions. The relatively slight degree of conjugation observed with adenosine (Table I) may be due to bonds involving the primary hydroxyl group at the 5' position. Thymidine displayed a reactivity of similar magnitude.

DISCUSSION

From the results described above it is evident that carbodiimides afford a means of conjugating nucleotides and oligonucleotides to proteins. Evidence has been presented which indicates that the bulk of the reaction takes place with protein amino groups. It would also appear that denatured DNA forms a stable bond with proteins under similar conditions. However, the nature of the bond here is not established. It is possible that a portion of the binding involves functional groups on purine and pyrimidine bases rather than terminal phosphate groups.

The natural occurrence of N—P—O bonds in α -casein has been reported by Perlmann (15, 16).

On the basis of results of enzymatic digestion, she inferred that 40% of the total phosphorus was bound in an N—P—O linkage, 20% as pyrophosphate and 40% as a phosphomonoester. She found that complete removal of the phosphorus was accompanied by disintegration of the protein into smaller units.

In the past several years, several methods have been described for combining purine and pyrimidine bases or nucleosides with protein in order to render these materials antigenic. Earlier studies by Butler *et al.* and by Tanenbaum and Beiser employed trichloromethyl purines and pyrimidines for conjugation (17, 18). While our own studies were in progress, Erlanger and Beiser described a reaction involving the vicinal hydroxyl groups on the sugar moiety of ribonucleotides (19). The ribonucleotide was oxidized with sodium periodate, coupled to protein, and the linkage stabilized by reduction. This reaction converted the ribose five-membered ring to a six-membered ring containing a nitrogen derived from the protein. Another recent approach has been that of Sela *et al.* which involves the conversion of mononucleotides or nucleosides to nucleoside-5'-carboxylic acids. The nucleoside-5' carboxylic acid is then coupled to polyamino acids containing free ϵ -amino groups (20).

In the method of conjugation described in this article, mononucleotides, oligonucleotides, and DNA are coupled directly to protein. By contrast, the methods described in the preceding paragraph require some form of preliminary chemical modification of the mononucleotide, nucleoside or base. The applicability of these methods to coupling larger units (oligo- and polynucleotides) to proteins or polyamino acids has not been established. We believe, therefore, that the general method described herein has certain advantages over previously described procedures.

There are a variety of other phosphorus-containing compounds of biologic interest which might be linked to proteins in the presence of carbodiimides. Efforts to date to promote the formation of covalent conjugates between flavin mononucleotide and protein with EDC have not been conclusive, however. Clearly, more work is needed before the applicability of the coupling procedure to non-nucleotide monophosphates is established.

In the subsequent article we will describe studies on the antibody specificity of antisera obtained after immunization with these conjugates.

REFERENCES

1. Khorana, H. G., In *Some Recent Developments in the Chemistry of Phosphate Esters of Biological Interest*, p. 126, John Wiley & Sons, Inc., New York, 1961.
2. Halloran, M. J. and Parker, C. W., *J. Immun.*, **66**: 379, 1966.
3. Halloran, M. J. and Parker, C. W., *J. Lab. Clin. Med. (Abstr.)*, **64**: 865, 1964.
4. Gilham, P. T. and Khorana, H. G., *J. Amer. Chem. Soc.*, **80**: 6212, 1958.
5. Khorana, H. G. and Vizsolyi, J. P., *J. Amer. Chem. Soc.*, **83**: 675, 1961.
6. Michelson, A. M., *Biochim. Biophys. Acta*, **91**: 1, 1964.
7. Hurst, R. O., *Canad. J. Biochem.*, **42**: 287, 1964.
8. Parker, C. W., In *Methods in Medical Research*, Vol. 10, p. 192, Year Book Medical Publishers, Chicago, 1964.
9. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J., *J. Biol. Chem.*, **193**: 265, 1951.
10. Jacob, T. M. and Khorana, H. G., *J. Amer. Chem. Soc.*, **86**: 1630, 1964.
11. Starbuck, W. C. and Busch, H., *Biochim. Biophys. Acta*, **78**: 594, 1963.
12. Stuart, A. and Khorana, H. G., *J. Biol. Chem.*, **239**: 3885, 1964.
13. Heppel, L. A., In *Methods in Enzymology*, Vol. 2, p. 530, Academic Press, New York, 1955.
14. Gottschalk, A., Murphy, W. H. and Graham, E. R. B., *Nature*, **194**: 1051, 1962.
15. Perlmann, G. E., In *Phosphorus Metabolism*, Vol. 2, p. 167, The Johns Hopkins Press, Baltimore, 1952.
16. Perlmann, G. E., *Nature*, **174**: 273, 1954.
17. Butler, V. P., Jr., Beiser, S. M., Erlanger, B. F., Tanenbaum, S. W., Cohen, S. and Bendich, A., *Proc. Nat. Acad. Sci.*, **48**: 1597, 1962.
18. Tanenbaum, S. W. and Beiser, S. M., *Proc. Nat. Acad. Sci.*, **49**: 662, 1963.
19. Erlanger, B. F. and Beiser, S. M., *Proc. Nat. Acad. Sci.*, **52**: 68, 1964.
20. Sela, M., Ungar-Waron, H. and Shechter, Y., *Proc. Nat. Acad. Sci.*, **52**: 285, 1964.

THE PRODUCTION OF ANTIBODIES TO MONONUCLEOTIDES, OLIGONUCLEOTIDES AND DNA

MARK J. HALLORAN¹ AND CHARLES W. PARKER²

*From the Immunology Division, Department of Medicine, Washington University School of Medicine,
St. Louis, Missouri*

Received for publication August 2, 1965

In the accompanying article we have described methods for preparing mono, oligo and polynucleotide protein conjugates (1).³ This article is concerned with the evaluation of the immunologic response to these preparations. We will present evidence for the formation of antibodies with specificity for nucleotides and DNA as demonstrated by precipitin and complement fixation reactions. The results of preliminary evaluation of the antibody response to tetrathymidylic acid in terms of the number of nucleotide residues involved in the antigenic site also will be reported.

MATERIALS AND METHODS

Methods for preparing protein conjugates and tetrathymidylic acid are described in the preceding article (1).

Random bred albino rabbits (2.5 kg) were immunized with either 2 or 3 mg of nucleotide or polynucleotide-protein conjugate in complete Freund's adjuvant, distributed among the footpads. Antisera were obtained by cardiac puncture at 21 to 26 days. Globulin fractions were obtained by precipitation at 50% ammonium sulfate followed by dialysis.

In quantitative precipitin analysis, antigen and antisera or globulin fractions were incubated at 37°C for 1 hr, and at 4°C for 16 to 36 hr. Precipitates were washed three times with ice-cold

saline, dissolved in 0.5% sodium lauryl sulfate in water and read at 280 mμ on the spectrophotometer. In calculating the amount of antibody in the precipitate, a correction was made for antigen contribution (see legend, Fig. 1) and it was assumed that a 1-mg/ml solution of rabbit γ-globulin has an absorbance of 1.5 at 280 mμ.

Quantitative complement fixation using the 50% hemolytic unit was carried out as described in (2).

RESULTS

Eight antisera to T5'-PO₄-EDC-HSA were evaluated using T5'-PO₄-CMC-B₇G as the precipitating antigen. The amount of antibody precipitated varied from 0.6 to 1.2 mg/ml. An example of a precipitin curve with a globulin fraction and varying amounts of antigen is shown in Figure 1. The complement fixation curve of this same globulin fraction at a dilution of 1:100 with denatured DNA as antigen is shown in Figure 2. Under the same conditions native DNA failed to fix complement with this antiserum. The amounts of T5'-PO₄ specific antibody formed when T5'-PO₄-CMC-B₇G was used as the immunizing antigen were comparable to the above.

The results of hapten inhibition of precipitation with antibody to T5'-PO₄ are shown in Figure 3.⁴ The relatively poor inhibition by thymine and thymidine as compared to thymidylic acid indicates that antibody specificity is to the entire molecule. Mixtures of the component parts of the T5'-PO₄ molecule (e.g., 2-deoxy-d-ribose, thymine and inorganic phosphate) inhibited only slightly better than thymine alone.

⁴ Using T5'-PO₄-CMC-B₇G as precipitating antigen at 0.06 M T5'-PO₄, more than 90% inhibition of precipitation was observed. This curve was not shown because a small amount of spontaneous precipitation by the antigen resulted in higher blanks.

¹ Postdoctoral Fellow of the United States Public Health Service, Grant 2 T1-AI-219.

² Recipient of a Research Career Development Award from the United States Public Health Service.

³ For abbreviations see footnote¹ in the preceding article (1). Protein conjugates are designated by nucleotide, coupling agent and protein. For example T5'-PO₄-EDC-B₇G was obtained by conjugation of thymidylic acid to B₇G in the presence of 1-ethyl-3-diisopropylaminocarbodiimide (EDC).

Adenylate and cytidylate inhibited much less effectively than $T5'-PO_4$.

The specificity of the thymidylate inhibition of precipitation was evaluated with two unrelated antigen-antibody systems. No inhibition was seen at concentrations as high as 0.06 M with egg albumin and CMC-B γ G and their respective antibodies.

Similarly, hapten inhibition of complement fixation between antibody to $T5'-PO_4$ -EDC-B γ G and $T5'-PO_4$ -EDC-HSA could be demonstrated (Fig. 4). The relative inhibitory capacity of various haptens was similar to that in hapten inhibition of precipitation; as expected, lower concentrations of hapten were required for 50% inhibition in the complement fixation system. No complement fixation was observed between EDC-HSA and the anti $T5'-PO_4$ -EDC-B γ G globulin fraction. This is in accord with nearly complete inhibition of complement fixation by $T5'-PO_4$ at a concentration of 3×10^{-5} M. No complement fixation was observed between $T5'-PO_4$ -EDC-HSA and "normal" rabbit globulin.

The specificity of inhibition of complement fixation by $T5'-PO_4$ was evaluated with penicoyl-B γ G (3) and its specific rabbit antiserum (in the form of a globulin fraction). No inhibition of complement fixation was observed at a $T5'-PO_4$ concentration of 0.06 M.

Antisera to tetrathymidylate acid. Three antisera were obtained to $(T5')_4$ -EDC-HSA. On the basis of the amount of globulin precipitated with $T5'-PO_4$ -CMC-B γ G the sera contained an average of 1.0 mg/ml antibody specific for the thymidylate polymer. The results of hapten inhibition of a pooled globulin fraction of the three sera are shown in Table I. The monomer and the tetramer of thymidylate were about equivalent as inhibitors (based on the number of thymidylate residues present). Higher concentrations of hapten were not used because of the limited supply of tetrathymidylate. Similar inhibition data were obtained with an antibody specific for $T5'-PO_4$ -CMC-HSA. The somewhat lower efficiency of the tetramer as an inhibitor using equivalent concentrations of thymidylate residues presumably is due to steric hindrance to

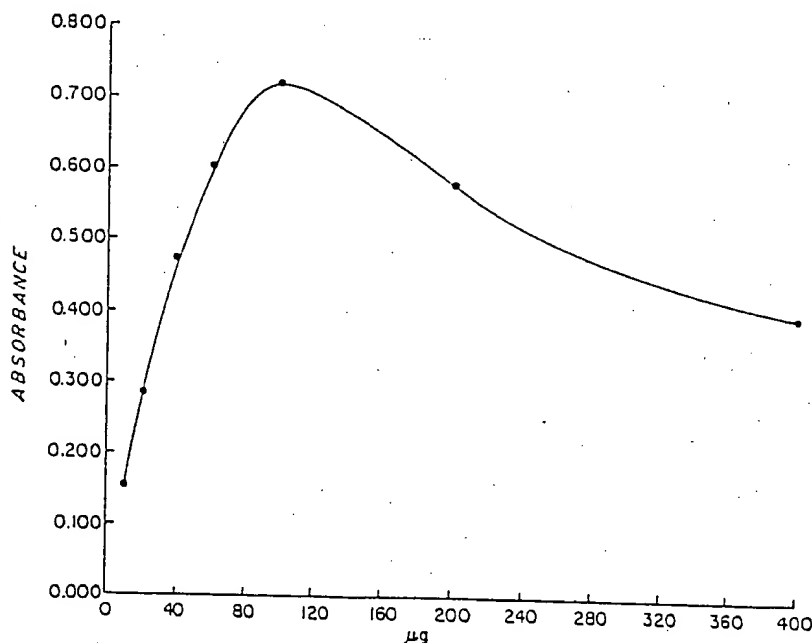


Figure 1. Analysis of rabbit antiserum to $T5'-PO_4$ -EDC-HSA using $T5'-PO_4$ -CMC-B γ G as precipitating antigen. The indicated amounts of antigen (abscissa) were incubated with 0.5-ml volumes of serum (duplicate tubes). Precipitates were washed and analyzed in a volume of 1.0 ml 0.5% sodium lauryl sulfate as described in the text. The absorbance at 280 m μ (ordinate) is corrected for antigen contribution assuming complete precipitation of antigen in antibody excess, 95% at equivalence, and 80% in antigen excess.

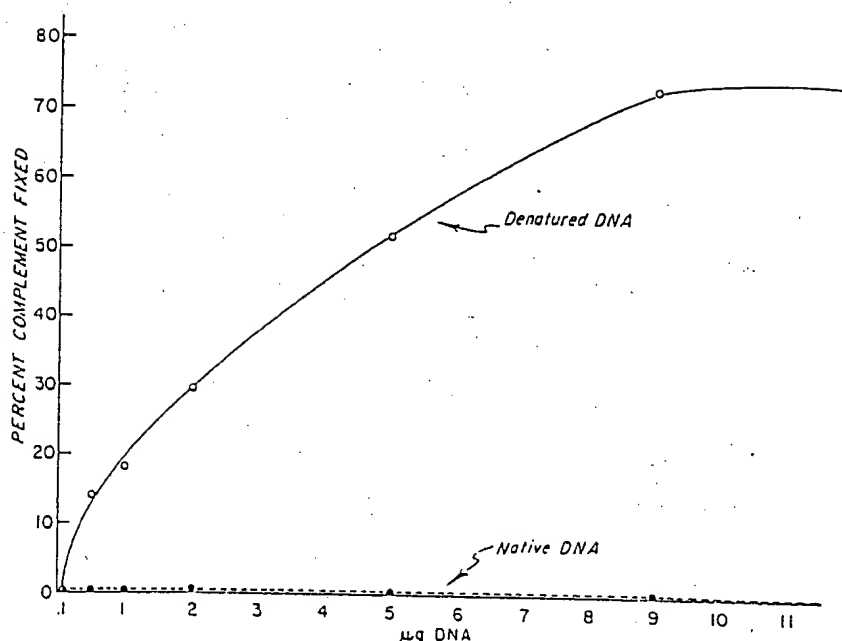


Figure 2. Complement fixation: Analysis of a rabbit antiserum to T5'-PO₄-EDC-HSA using native and denatured DNA as antigens. The indicated amounts of DNA (abscissa) were incubated with a 1:100 dilution of a globulin fraction of the above antiserum. No complement was fixed in antigen and antibody control tubes. Denatured DNA did not fix complement with "normal" rabbit globulin (obtained from animals immunized with unrelated antigens in complete adjuvant).

the simultaneous binding of several antibody molecules to the same tetra-thymidylate molecule.

Antisera to DNA. Antisera to DNA-protein conjugates failed to precipitate with homologous and heterologous DNA by ring test and double diffusion in agar gel. Antisera both to native Figure 5B and denatured Figure 5A calf thymus DNA antiserum (immunization with DNA-CMC-B γ G) fixed complement with denatured calf thymus DNA at dilutions of 1:100 or higher. No complement was fixed with native DNA. Antisera to salmon sperm and calf thymus DNA fixed complement equally well with homologous and heterologous DNA. This result suggested that complement fixation was not due to protein antigens contaminating the DNA preparations. The presence of antibodies with nucleotide specificity was confirmed by the results of precipitin analysis. Antisera to the DNA-CMC-B γ G conjugates formed precipitates with T5'-PO₄-EDC-HSA (Fig. 6). The average serum concentration of nucleotide specific antibody was estimated to be 1.2 mg/ml, using an antibody to salmon sperm DNA (immunization with DNA-CMC-B γ G) and the above precipitating antigen. Precipitation was inhibited to the extent of 20%, 35% and 80%

at T5'-PO₄ concentrations of 0.006, 0.03 and 0.06 M respectively. Deoxyadenylic acid produced comparable inhibition, in contrast to what was observed with antibodies to T5'-PO₄, suggesting that both deoxyribonucleotides (and presumably all four) participate in the antigenic groupings.

Antibody with nucleotide specificity at a concentration of 0.8 to 1.0 mg/ml also was demonstrated in the serum from two animals immunized with a mock DNA-EDC protein conjugate. The protein had been reacted with EDC in the absence of DNA, and then dialyzed thoroughly to remove unreacted coupling agent. On mixing the EDC-protein with denatured DNA at neutral pH a precipitate formed which then was used for immunization.

DISCUSSION

The results of the immunologic studies indicate that nucleotides, oligonucleotides, and DNA-protein conjugates induce the formation of antibodies with nucleotide specificity. The antibodies react both with denatured DNA and with nucleotide protein conjugates. While the immunologic response to analogous RNA protein preparations

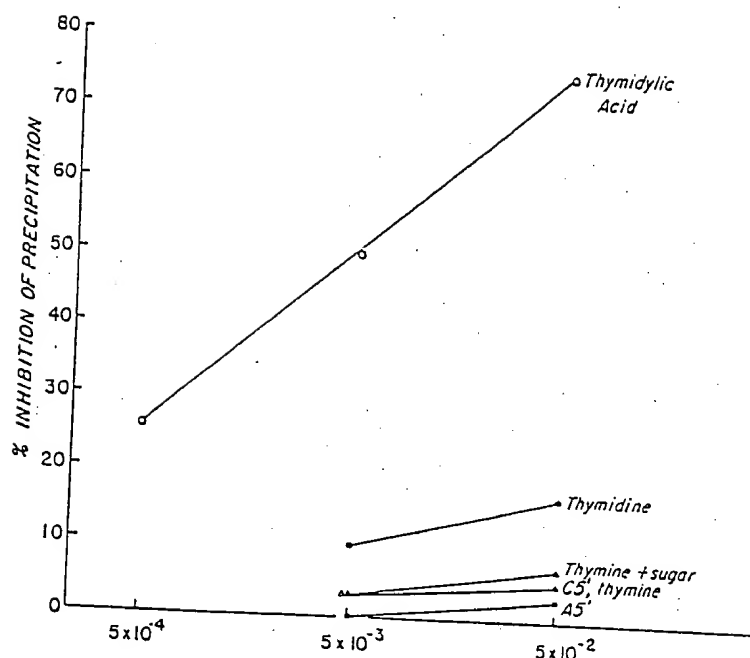


Figure 3. Hapten inhibition of precipitation of a globulin fraction of rabbit antiserum to T5'-PO₄-EDC-B₇G using T5'-PO₄-EDC-HSA as the precipitating antigen (at equivalence, 0.1 mg antigen protein). Each point represents the result of duplicate determinations. The deviation in absorbance between duplicate tubes did not exceed 3%. Control precipitates (no hapten) contained 0.2 mg antibody protein. The figures on the abscissa indicate the final hapten concentration in mM/ml. Analysis of precipitates was carried out as described in the text and the legend to Figure 1. (O—O = thymidylic acid; ●—● = thymidine; △—△ = thymine + deoxyribose (each at the concentration indicated on the abscissa); ▲—▲ = cytidylic or thymine; ■—■ = deoxyadenylic acid.) Thymidylic acid did not inhibit precipitation at 0.06 M in two unrelated antigen antibody systems.

has not been studied, it may be presumed that antibodies to the different types of RNA could be obtained by the same procedure.

In employing water soluble carbodiimides to prepare nucleotide protein conjugates several side reactions can take place. Carbodiimides promote the formation of peptide bonds between protein molecules (4), altering the antigenic structure of the protein. Carbodiimides can be substituted on the protein (5) or on nucleotide bases (6) directly under certain conditions, introducing new antigenic groups. Any contribution of the protein and the coupling agent to the antibody response can be evaluated by nucleotide inhibition of precipitation and complement fixation. Moreover, contributions by non-nucleotide antibodies can be largely or completely eliminated by the use of unconjugated DNA as the antigen in complement fixation, by varying the protein and the coupling agent used in preparing the test antigen, or by absorption of the antiserum.

In the past several years several groups have reported antibody formation to purine and pyrimidine bases, nucleosides or mononucleotides (7-10). The production of antibodies to oligonucleotides and to purified DNA has remained a problem, however. Yachnin was unable to produce antibodies to homopolymers of several nucleotides or to purified DNA (11, 12). During the course of our own studies Plescia *et al.* reported that the precipitate formed by mixing the polycationic protein, methylated serum albumin and denatured DNA produced antibodies reactive with DNA (13). The basis for the immunogenicity of DNA and other acidic polymers, when given in the form of a reversible complex with methylated serum albumin is not entirely clear. In addition to the several explanations suggested by Plescia *et al.*, we believe there is the possibility that the ester groups on the protein might react *in vivo* with formation of covalent bonds between DNA and protein. Fruton has called attention to the fact that

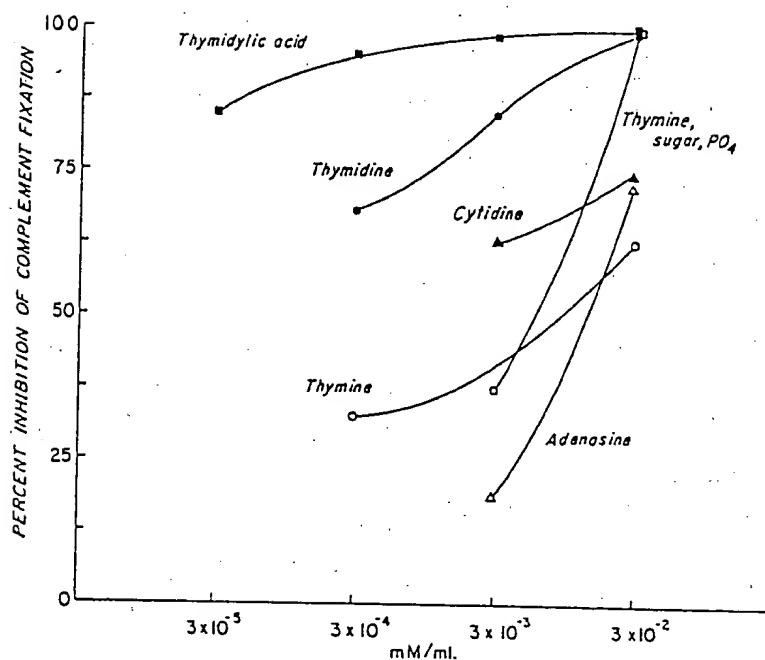


Figure 4. Hapten inhibition of complement fixation. A 1:100 dilution of a globulin fraction of rabbit anti-T5'-PO₄-EDC-HSA was incubated with 1 μ g T5'-PO₄-EDC-B γ G. Hapten concentrations are expressed in mmoles/ml (abscissa). (■—■ = thymidylic acid; ●—● = thymidine; ▲—▲ = cytidine; ○—○ = thymine; △—△ = adenosine; □—□ = a mixture of thymine, 2-deoxy-d-ribose, and inorganic phosphate, each at the concentrations indicated on the abscissa).

TABLE I
Comparative hapten inhibition of precipitation of globulin fractions from animals immunized with mono- and tetra-thymidylate

Immunizing Antigen	Concentration of Hapten	Hapten	
		T5'-PO ₄	(T5') ₄ ^a
		%	%
HSA-(T5') ₄ -EDC	6×10^{-4}	13	10
	6×10^{-5}	5	0
HSA-T5'-PO ₄ -EDC	6×10^{-4}	32	17
	6×10^{-5}	17	14

^a The molarity of the (T5')₄ hapten is expressed per T5'-PO₄ residue.

The precipitating antigen used for both antibodies was the equivalent amount of T5'-PO₄-CMC-B γ G.

Control precipitates (no hapten present) contained 0.16 mg antibody protein. Precipitates were analyzed in duplicate as described in the text and the legend for Figure 1.

aliphatic esters of amino acids are activated in a thermodynamic sense and might undergo reactions such as aminolysis *in vivo* (14). In this regard it would be of interest to study the im-

munogenicity of DNA complexed with other polycationic macromolecules. The formation of antibodies to the insoluble mock conjugate of EDC-treated protein and DNA which we observed in this study is presumably analogous to what occurs with the methylated serum albumin-DNA complexes.

The relative advantages of the carbodiimide and the methylated serum albumin methods for producing antibodies to DNA and oligonucleotides remain to be fully evaluated. The carbodiimide technique is applicable to units of any size; the minimal nucleotide size for antigenicity in oligonucleotide-methylated serum albumin complexes is probably of the order of four to eight nucleotide residues and may be larger in some instances (15). Moreover, the conjugates prepared by the carbodiimide procedure usually are entirely soluble; this is an advantage in that in the methylated serum albumin-DNA insoluble complex, large portions of the DNA may be buried and unable to act as antigenic sites. On the other hand, carbodiimides which contain secondary and tertiary amine groups can react with nucleotide bases altering DNA structure. For this reason it may be desirable to use the

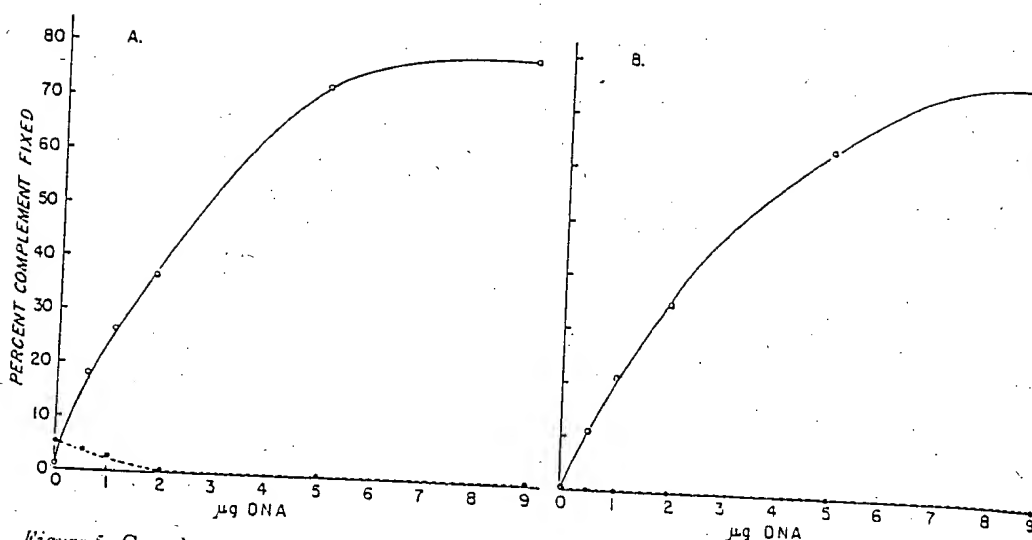


Figure 6. Complement fixation by a 1:100 dilution of a globulin fraction of rabbit antiserum to native (curve B) and denatured (curve A) calf thymus DNA (immunized with DNA-CMC-B₇C). The indicated amounts of native (●-----●) and denatured (O—O) calf thymus DNA were used as the complement-fixing antigen.

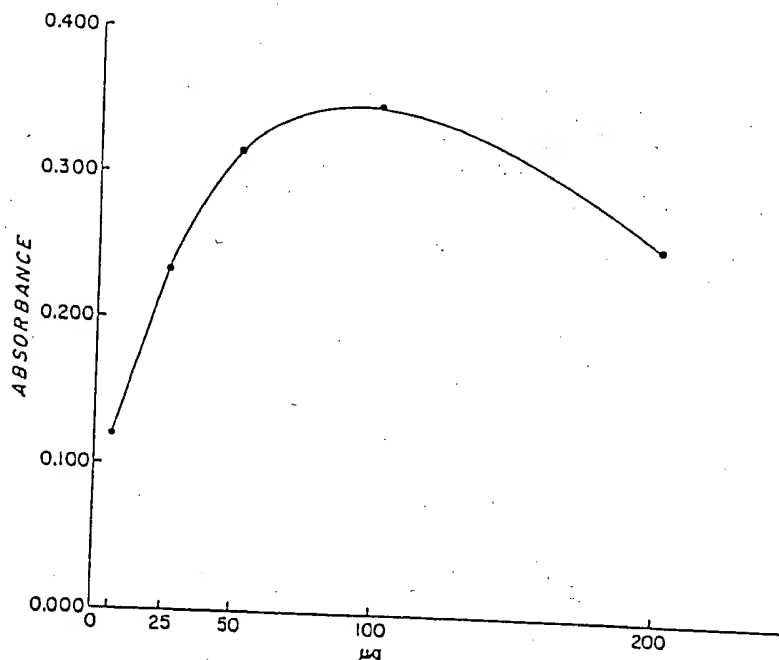


Figure 6. Analysis of rabbit antiserum to denatured calf thymus DNA-CMC-B₇C using T5'-PO₄-EDC-HSA as precipitating antigen. The indicated amounts of antigen protein (abscissa) were incubated with 0.2-ml volumes of antiserum in duplicate tubes. Absorbance (ordinate) is that of dissolved precipitate in 1.0 ml 0.5% sodium lauryl sulfate and is corrected for reading due to antigen (see text and legend to Figure 1).

nonpolar carbodiimide, DCC, as the coupling agent rather than EDC or CMC.

The question remains as to whether antibodies to various types of DNA will be able to distin-

guish the homologous antigen from heterologous DNA. The limited number of different nucleotide bases and the structural similarity between the two purines and the two pyrimidines make

the degree of potential antigenic variation in single stranded DNA much less than that in a protein. Presumptive evidence that an antibody site may encompass at least 4 to 5 nucleotide residues has been obtained in the studies of Stollar, Levine and their colleagues (16) with sera from patients with lupus erythematosus. They found several sera in which a tetramer or pentamer of thymidylic acid inhibited complement fixation much more effectively than did the monomer. Our own preliminary studies using tetrathymidylate as antigen have not provided evidence for an antibody response to the entire oligonucleotide. Nor were there obvious differences between antibodies to two types of DNA (differing appreciably in base composition) in terms of their ability to fix complement fixation with the homologous and heterologous DNA. It seems likely, however, that the antibody responses do differ and that this might be demonstrated by absorption of the antisera with the heterologous DNA. Similarly, absorption of the antisera to tetrathymidylic acid might reveal that a portion of the antibody had specificity for the entire oligonucleotide.

REFERENCES

1. Halloran, M. J. and Parker, C. W., *J. Immun.*, **96**: 373, 1966.
2. Kabat, E. A. and Mayer, M. M., In *Experimental Immunochimistry*, 2nd ed., p. 133, Charles C Thomas, Springfield, Ill., 1961.
3. Thiel, J. A., Mitchell, S. and Parker, C. W., *J. Allergy*, **35**: 399, 1964.
4. Sheehan, J. C. and Hlavka, J. J., *J. Org. Chem.*, **21**: 439, 1956.
5. Goodfriend, T. L., Levine, L. and Fasman, G. D., *Science*, **144**: 1344, 1964.
6. Augusti-Tocco, G. and Brown, G. L., *Nature*, **206**: 683, 1965.
7. Butler, V. P., Jr., Beiser, S. M., Erlanger, B. F., Tanenbaum, S. W., Cohen, S. and Bendich, A., *Proc. Nat. Acad. Sci.*, **48**: 1597, 1962.
8. Tanenbaum, S. W. and Beiser, S. M., *Proc. Nat. Acad. Sci.*, **49**: 662, 1963.
9. Erlanger, B. F. and Beiser, S. M., *Proc. Nat. Acad. Sci.*, **52**: 68, 1964.
10. Sela, M., Ungar-Waron, H. and Shechter, Y., *Proc. Nat. Acad. Sci.*, **52**: 285, 1964.
11. Yachnin, S., *Nature*, **195**: 1319, 1962.
12. Yachnin, S., *J. Immun.*, **91**: 528, 1963.
13. Plescia, O. J., Braun, W. and Paleczuk, N. C., *Proc. Nat. Acad. Sci.*, **52**: 279, 1964.
14. Fruton, J. S., In *The Proteins*, Edited by H. Neurath, 2nd ed., Vol. 1, p. 190, Academic Press, New York, 1964.
15. Plescia, O. J., Paleczuk, N. C., Braun, W. and Cora-Figueroa, E., *Science*, **148**: 1102, 1965.
16. Stollar, D., Levine, L., Lehrer, H. I. and Van Vunakis, H., *Proc. Nat. Acad. Sci.*, **48**: 874, 1962.

ENGELHARDT ET AL., U.S. PAT. APPL. SER. NO. 08/479,997
NEW CLAIMS 576-825 FOR SUBMISSION WITH AMENDMENT
(In Response to November 26, 2001 Office Action)

576. An oligo- or polydeoxyribonucleotide which is complementary to a nucleic acid of interest or a portion thereof, said oligo- or polydeoxyribonucleotide comprising at least one modified nucleotide having the formula

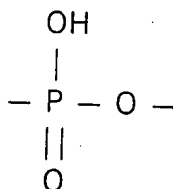


wherein PM is a phosphate moiety, SM is a sugar moiety and BASE is a base moiety selected from the group consisting of a pyrimidine, a purine and a deazapurine, or analog thereof, said PM being attached to SM, said BASE being attached to SM, and Sig being covalently attached to PM directly or through a chemical linkage, said Sig comprising a non-polypeptide, non-radioactive label moiety which can be directly or indirectly detected when attached to PM or when said modified nucleotide is incorporated into said oligo- or polydeoxyribonucleotide or when said oligo- or polydeoxyribonucleotide is hybridized to said complementary nucleic acid of interest or a portion thereof.

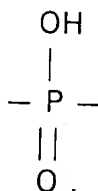
577. The oligo- or polydeoxyribonucleotide of claim 576, wherein said Sig is or renders the nucleotide or the oligo- or polydeoxyribonucleotide self-signaling or self-indicating or self-detecting.

578. The oligo- or polydeoxyribonucleotide of claim 576, wherein said Sig moiety comprises at least three carbon atoms.

579. The oligo- or polydeoxyribonucleotide of claim 576, wherein said covalent attachment is selected from the group consisting of



and

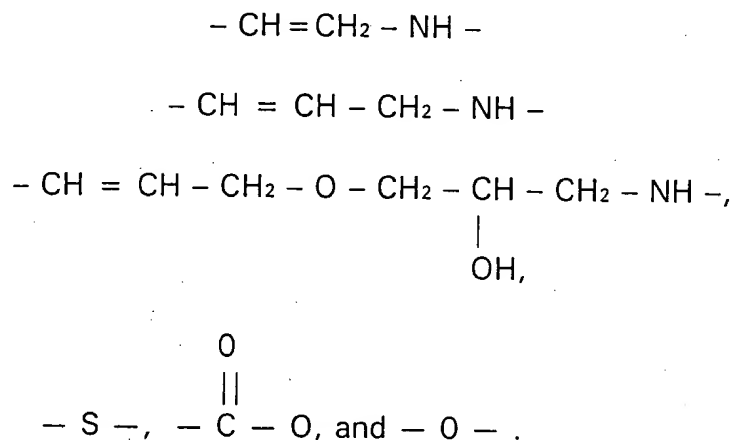


580. The oligo- or polydeoxyribonucleotide of claim 576, wherein said chemical linkage does not interfere substantially with the characteristic ability of Sig to form a detectable signal.

581. The oligo- or polydeoxyribonucleotide of claim 576, wherein said chemical linkage comprises a member selected from the group consisting of an olefinic bond at the α -position relative to the point of attachment to the nucleotide, a $-\text{CH}_2\text{NH}-$ moiety, or both.

582. The oligo- or polydeoxyribonucleotide of claim 576, wherein said chemical linkage comprises an allylamine group.

583. The oligo- or polydeoxyribonucleotide of claim 576, wherein said chemical linkage comprises or includes an olefinic bond at the α -position relative to the point of attachment to the nucleotide, or any of the moieties:



584. The oligo- or polydeoxyribonucleotide of claim 576, wherein said chemical linkage of Sig includes a glycosidic linkage moiety.

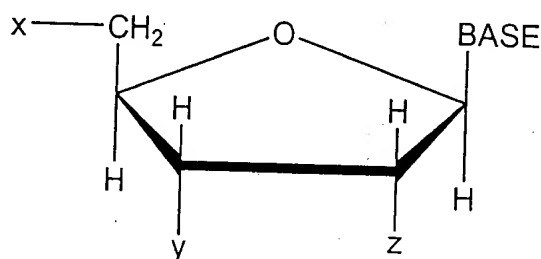
585. The oligo- or polydeoxyribonucleotide of claim 576, wherein said PM is monophosphate, a diphosphate or a triphosphate and said Sig moiety is covalently attached to said PM through a phosphorus atom or phosphate oxygen.

586. The oligo- or polydeoxyribonucleotide of claim 576, wherein Sig comprises a component selected from the group consisting of biotin, iminobiotin, an electron dense component, a magnetic component, a metal-containing component, a fluorescent component, a chemiluminescent component, a chromogenic component or a combination of any of the foregoing.

587. The oligo- or polydeoxyribonucleotide of claim 586, wherein said electron dense component comprises ferritin.

588. The oligo- or polydeoxyribonucleotide of claim 586, wherein said magnetic component comprises magnetic oxide.
589. The oligo- or polydeoxyribonucleotide of claim 588, wherein said magnetic oxide comprises ferric oxide.
590. The oligo- or polydeoxyribonucleotide of claim 586, wherein said metal-containing component is catalytic.
591. The oligo- or polydeoxyribonucleotide of claim 586, wherein said fluorescent component comprises a member selected from the group consisting of fluorescein, rhodamine and dansyl.
592. The oligo- or polydeoxyribonucleotide of claim 576, wherein said Sig moiety is attached to a terminal nucleotide in said oligo- or polydeoxyribonucleotide.
593. The oligo- or polydeoxyribonucleotide of claim 592, wherein the sugar moiety of said terminal nucleotide has a hydrogen atom at the 2' position thereof.
594. The oligo- or polydeoxyribonucleotide of claim 592, wherein the sugar moiety of said terminal nucleotide has oxygen atoms at each of the 2' and 3' positions thereof.
595. The oligo- or polydeoxyribonucleotide of claim 576, comprising at least one ribonucleotide.

596. An oligo- or polydeoxyribonucleotide which is complementary to a nucleic acid of interest or a portion thereof, said oligo- or polydeoxyribonucleotide comprising at least one modified nucleotide having the structural formula:



wherein BASE is a moiety selected from the group consisting of a pyrimidine, a purine and a deazapurine, or analog thereof, and wherein BASE is attached to the 1' position of the pentose ring from the N1 position when BASE is a pyrimidine or from the N9 position when BASE is a purine or a deazapurine;

wherein x is selected from the group consisting of $\text{H}-$, $\text{HO}-$, a mono-phosphate, a di-phosphate and a tri-phosphate;

wherein y is selected from the group consisting of $\text{H}-$, $\text{HO}-$, a mono-phosphate, a di-phosphate and a tri-phosphate;

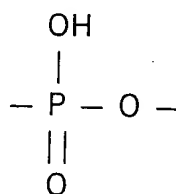
wherein z is selected from the group consisting of $\text{H}-$, $\text{HO}-$, a mono-phosphate, a di-phosphate and a tri-phosphate; and

wherein Sig is covalently attached directly or through a chemical linkage to at least one phosphate selected from the group consisting of x, y, z, and a combination thereof, said Sig comprising a non-polypeptide, non-radioactive label moiety which can be directly or indirectly detected when so attached to said phosphate or when said modified nucleotide is incorporated into said oligo- or polydeoxyribonucleotide or when said oligo- or polydeoxyribonucleotide is hybridized to said complementary nucleic acid of interest or a portion thereof.

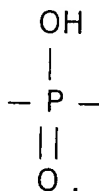
597. The oligo- or polydeoxyribonucleotide of claim 596, wherein said Sig is or renders the nucleotide or the oligo- or polydeoxyribonucleotide self-signaling or self-indicating or self-detecting.

598. The oligo- or polydeoxyribonucleotide of claim 596, wherein said Sig moiety comprises at least three carbon atoms.

599. The oligo- or polydeoxyribonucleotide of claim 596, wherein said covalent attachment is selected from the group consisting of



and

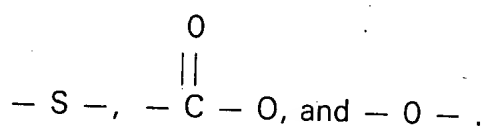
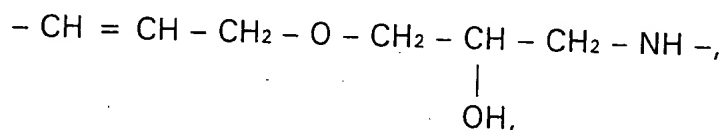
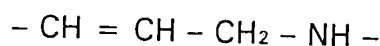


600. The oligo- or polydeoxyribonucleotide of claim 596, wherein said chemical linkage does not interfere substantially with the characteristic ability of Sig to form a detectable signal.

601. The oligo- or polydeoxyribonucleotide of claim 596, wherein said chemical linkage comprises a member selected from the group consisting of an olefinic bond at the α -position relative to the point of attachment to the nucleotide, a $-\text{CH}_2\text{NH}-$ moiety, or both.

602. The oligo- or polydeoxyribonucleotide of claim 596, wherein said chemical linkage comprises an allylamine group.

603. The oligo- or polydeoxyribonucleotide of claim 596, wherein said chemical linkage comprises or includes an olefinic bond at the α -position relative to the point of attachment to x, y or z, or any of the moieties:



604. The oligo- or polydeoxyribonucleotide of claim 596, wherein said chemical linkage of Sig includes a glycosidic linkage moiety.

605. The oligo- or polydeoxyribonucleotide of claim 596, wherein said x and y each comprise a member selected from the group consisting of a monophosphate, a diphosphate and a triphosphate and said Sig moiety is covalently attached to either or both of said x and y through a phosphorus atom or phosphate oxygen.

606. The oligo- or polydeoxyribonucleotide of claim 596, wherein Sig comprises a component selected from the group consisting of biotin, iminobiotin, an electron dense component, a magnetic component, a metal-containing component, a fluorescent component, a chemiluminescent component, a chromogenic component or a combination of any of the foregoing.

607. The oligo- or polydeoxyribonucleotide of claim 606, wherein said electron dense component comprises ferritin.

608. The oligo- or polydeoxyribonucleotide of claim 606, wherein said magnetic component comprises magnetic oxide.

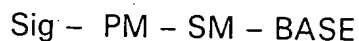
609. The oligo- or polydeoxyribonucleotide of claim 608, wherein said magnetic oxide comprises ferric oxide.

610. The oligo- or polydeoxyribonucleotide of claim 606, wherein said metal-containing component is catalytic.

611. The oligo- or polydeoxyribonucleotide of claim 606, wherein said fluorescent component comprises a member selected from the group consisting of fluorescein, rhodamine and dansyl.

612. The oligo- or polydeoxyribonucleotide of claim 596, wherein said Sig moiety is attached to a terminal nucleotide in said oligo- or polydeoxyribonucleotide.

617. An oligo- or polynucleotide which is complementary to a nucleic acid of interest or a portion thereof, said oligo- or polynucleotide comprising at least one modified nucleotide having the formula

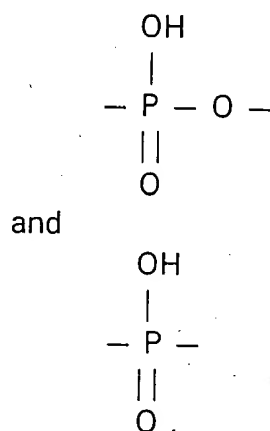


wherein PM is a phosphate moiety, SM is a sugar moiety and BASE is a moiety selected from the group consisting of a pyrimidine, a purine and a deazapurine, or analog thereof, said PM being attached to SM, said BASE being attached to SM, and Sig being covalently attached to PM directly or via a chemical linkage, said Sig comprising a non-polypeptide, non-radioactive label moiety which can be directly or indirectly detected when attached to PM or when said modified nucleotide is incorporated into said oligo- or polynucleotide, or when said oligo- or polynucleotide is hybridized to said complementary nucleic acid of interest or a portion thereof, provided that when said oligo- or polynucleotide is an oligoribonucleotide or a polyribonucleotide, and when Sig is attached through a chemical linkage to a terminal PM at the 3' position of a terminal ribonucleotide, said chemical linkage is not obtained through a 2',3' vicinal oxidation of a 3' terminal ribonucleotide previously attached to said oligoribonucleotide or polyribonucleotide.

618. The oligo- or polynucleotide of claim 617, wherein said Sig is or renders the nucleotide or the oligo- or polynucleotide self-signaling or self-indicating or self-detecting.

619. The oligo- or polynucleotide of claim 617, wherein said Sig moiety comprises at least three carbon atoms.

620. The oligo- or polynucleotide of claim 617, wherein said covalent attachment is selected from the group consisting of

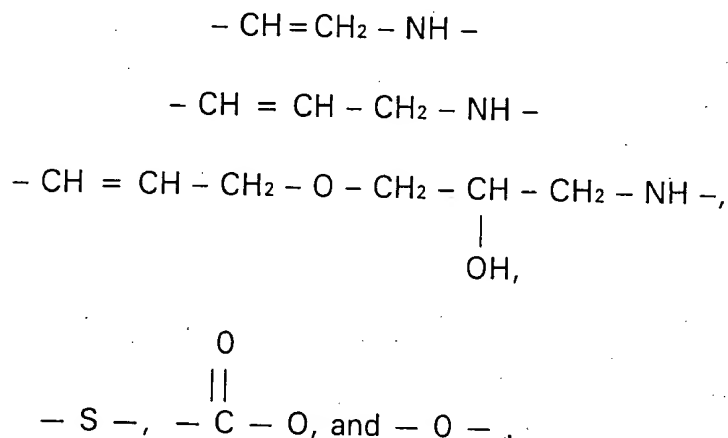


621. The oligo- or polynucleotide of claim 617, wherein said chemical linkage does not interfere substantially with the characteristic ability of Sig to form a detectable signal.

622. The oligo- or polynucleotide of claim 617, wherein said chemical linkage comprises a member selected from the group consisting of an olefinic bond at the α -position relative to the point of attachment to the nucleotide, a $-\text{CH}_2\text{NH}-$ moiety, or both.

623. The oligo- or polynucleotide of claim 617, wherein said chemical linkage comprises an allylamine group.

624. The oligo- or polynucleotide of claim 617, wherein said chemical linkage comprises or includes an olefinic bond at the α -position relative to the point of attachment to the nucleotide, or any of the moieties:



625. The oligo- or polynucleotide of claim 617, wherein said chemical linkage of Sig includes a glycosidic linkage moiety.

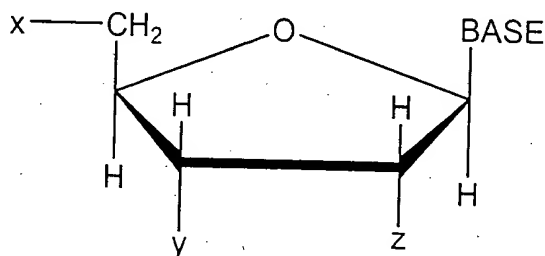
626. The oligo- or polynucleotide of claim 617, wherein said PM is a monophosphate, a diphosphate or a triphosphate and said Sig moiety is covalently attached to said PM through a phosphorus atom or a phosphate oxygen.

627. The oligo- or polynucleotide of claim 617, wherein Sig comprises a component selected from the group consisting of biotin, iminobiotin, an electron dense component, a magnetic component, a metal-containing component, a fluorescent component, a chemiluminescent component, a chromogenic component or a combination of any of the foregoing.

628. The oligo- or polynucleotide of claim 627, wherein said electron dense component comprises ferritin.

629. The oligo- or polynucleotide of claim 627, wherein said magnetic component comprises magnetic oxide.
630. The oligo- or polynucleotide of claim 629, wherein said magnetic oxide comprises ferric oxide.
631. The oligo- or polynucleotide of claim 627, wherein said metal-containing component is catalytic.
632. The oligo- or polynucleotide of claim 627, wherein said fluorescent component comprises a member selected from the group consisting of fluorescein, rhodamine and dansyl.
633. The oligo- or polynucleotide of claim 617, wherein said Sig moiety is attached to a terminal nucleotide in said oligo- or polynucleotide.
634. The oligo- or polynucleotide of claim 633, wherein the sugar moiety of said terminal nucleotide has a hydrogen atom at the 2' position thereof.
635. The oligo- or polynucleotide of claim 633, wherein the sugar moiety of said terminal nucleotide has an oxygen atom at each of the 2' and 3' positions thereof.
636. The oligo- or polynucleotide of claim 617, comprising at least one deoxyribonucleotide.

637. An oligo- or polynucleotide which is complementary to a nucleic acid of interest or a portion thereof, said oligo- or polynucleotide comprising at least one modified nucleotide having the structural formula:



wherein BASE is a moiety selected from the group consisting of a pyrimidine, a purine and a deazapurine, or analog thereof, and wherein BASE is attached to the 1' position of the pentose ring from the N1 position when BASE is a pyrimidine or from the N9 position when BASE is a purine or a deazapurine;

wherein x is selected from the group consisting of H—, HO—, a mono-phosphate, a di-phosphate and a tri-phosphate;

wherein y is selected from the group consisting of H—, HO—, a mono-phosphate, a di-phosphate and a tri-phosphate;

wherein z is selected from the group consisting of H—, HO—, a mono-phosphate, a di-phosphate and a tri-phosphate; and

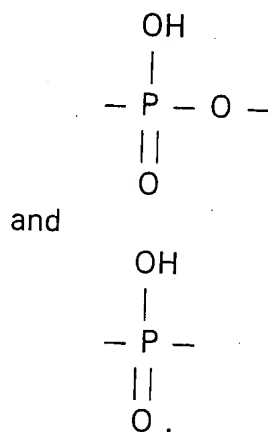
wherein Sig is covalently attached directly or through a chemical linkage to at least one phosphate selected from the group consisting of x, y and z, and a combination thereof, said Sig comprising a non-polypeptide, non-radioactive label moiety which can be directly or indirectly detected when so attached to said phosphate or when said modified nucleotide is incorporated into said oligo- or polynucleotide, or when said oligo- or polynucleotide is hybridized to said complementary nucleic acid of interest or a portion thereof, provided that when said oligo- or polynucleotide is an oligoribonucleotide or a polyribonucleotide and

when Sig is attached through a chemical linkage to a terminal PM at the 3' position of a terminal ribonucleotide, said chemical linkage is not obtained through a 2',3' vicinal oxidation of a 3' terminal ribonucleotide previously attached to said oligoribonucleotide or polyribonucleotide.

638. The oligo- or polynucleotide of claim 637, wherein said Sig is or renders the nucleotide or the oligo- or polynucleotide self-signaling or self-indicating or self-detecting.

639. The oligo- or polynucleotide of claim 637, wherein said Sig moiety comprises at least three carbon atoms.

640. The oligo- or polynucleotide of claim 637, wherein said covalent attachment is selected from the group consisting of

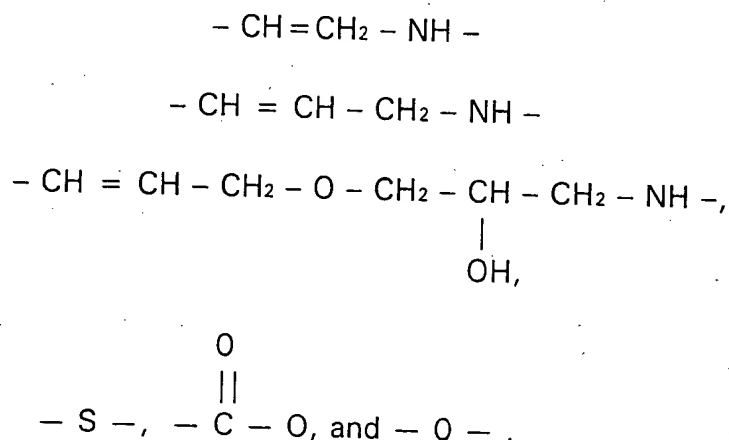


641. The oligo- or polynucleotide of claim 637, wherein said chemical linkage does not interfere substantially with the characteristic ability of Sig to form a detectable signal.

642. The oligo- or polynucleotide of claim 637, wherein said chemical linkage comprises a member selected from the group consisting of an olefinic bond at the α -position relative to the point of attachment to the nucleotide, a $-\text{CH}_2\text{NH}-$ moiety, or both.

643. The oligo- or polynucleotide of claim 637, wherein said chemical linkage comprises an allylamine group.

644. The oligo- or polynucleotide of claim 637, wherein said chemical linkage comprises or includes an olefinic bond at the α -position relative to x, y or z, or any of the moieties:



645. The oligo- or polynucleotide of claim 637, wherein said chemical linkage of Sig includes a glycosidic linkage moiety.

646. The oligo- or polynucleotide of claim 637, wherein said x and y each comprise a member selected from the group consisting of a monophosphate, a diphosphate and a triphosphate and Sig moiety is covalently attached to either or both of said x and y through a phosphorus atom or a phosphate oxygen.

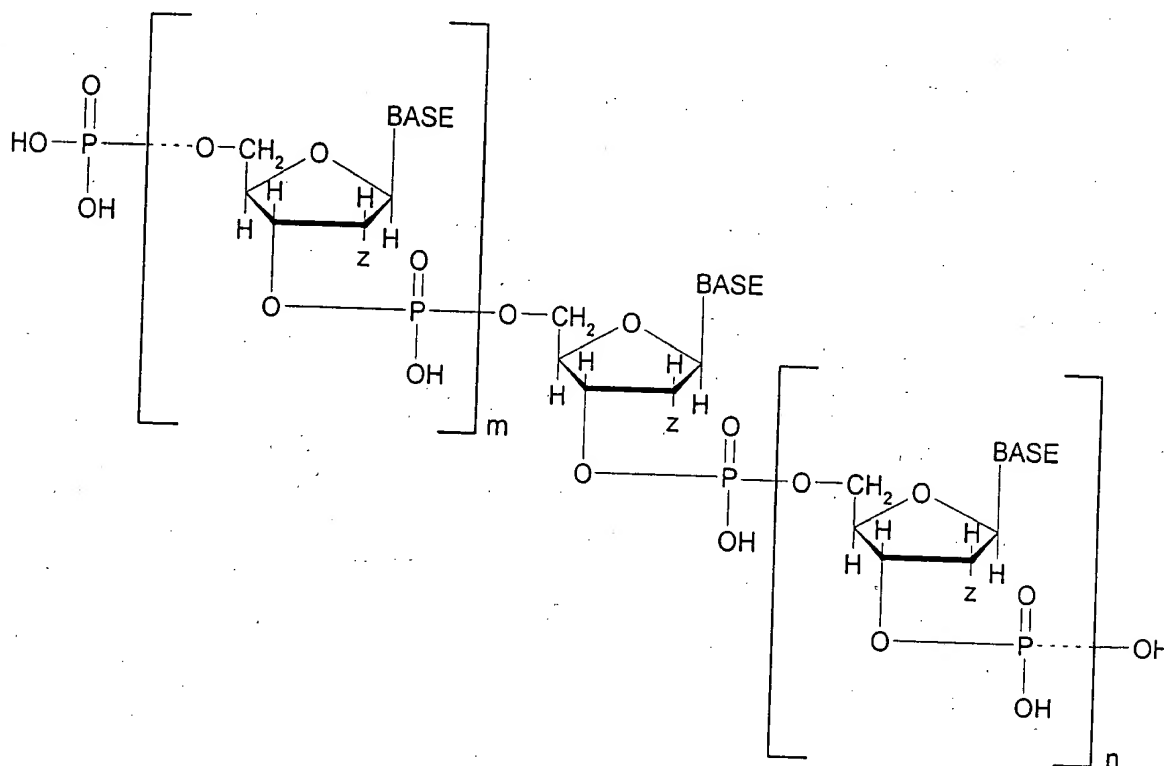
647. The oligo- or polynucleotide of claim 637, wherein Sig comprises a component selected from the group consisting of biotin, iminobiotin, an electron dense component, a magnetic component, a metal-containing component, a fluorescent component, a chemiluminescent component, a chromogenic component or a combination of any of the foregoing.
648. The oligo- or polynucleotide of claim 647, wherein said electron dense component comprises ferritin.
649. The oligo- or polynucleotide of claim 647, wherein said magnetic component comprises magnetic oxide.
650. The oligo- or polynucleotide of claim 649, wherein said magnetic oxide comprises ferric oxide.
651. The oligo- or polynucleotide of claim 647, wherein said metal-containing component is catalytic.
652. The oligo- or polynucleotide of claim 647, wherein said fluorescent component comprises a member selected from the group consisting of fluorescein, rhodamine and dansyl.
653. The oligo- or polynucleotide of claim 637, wherein said Sig moiety is attached to a terminal nucleotide in said oligo- or polynucleotide.

654. The oligo- or polynucleotide of claim 653, wherein z of said terminal nucleotide comprises a hydrogen atom at the 2' position thereof.

655. The oligo- or polynucleotide of claim 653, wherein both y and z of said terminal nucleotide comprise an oxygen atom at each of the 3' and 2' positions thereof, respectively.

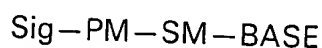
656. The oligo- or polynucleotide of claim 637, comprising at least one deoxyribonucleotide.

657. The oligo- or polynucleotide of claim 637, having the structural formula:



wherein m and n represent integers from 0 up to about 100,000, and wherein said Sig moiety is attached to at least one of the phosphate moieties in said structural formula.

658. An oligo- or polydeoxyribonucleotide which is complementary to a nucleic acid of interest or a portion thereof, said oligo- or polydeoxyribonucleotide comprising at least one modified nucleotide having the formula

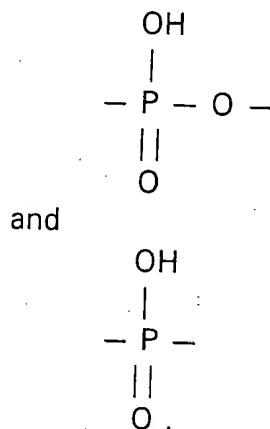


wherein PM is a phosphate moiety, SM is a sugar moiety and BASE is a base moiety selected from the group consisting of a pyrimidine, a purine and a deazapurine, or analog thereof, said PM being attached to SM, said BASE being attached to SM, and Sig being covalently attached to PM directly or through a chemical linkage, said Sig comprising a non-radioactive label moiety which can be directly or indirectly detected when attached to PM or when said modified nucleotide is incorporated into said oligo- or polydeoxyribonucleotide or when said oligo- or polydeoxyribonucleotide is hybridized to said complementary nucleic acid of interest or a portion thereof, and wherein Sig comprises biotin, iminobiotin, an electron dense component, a magnetic component, a metal-containing component, a fluorescent component, a chemiluminescent component, a chromogenic component or a combination of any of the foregoing.

659. The oligo- or polydeoxyribonucleotide of claim 658, wherein said Sig is or renders the nucleotide or the oligo- or polydeoxyribonucleotide self-signaling or self-indicating or self-detecting.

660. The oligo- or polydeoxyribonucleotide of claim 658, wherein said Sig moiety comprises at least three carbon atoms.

661. The oligo- or polydeoxyribonucleotide of claim 658, wherein said covalent attachment is selected from the group consisting of

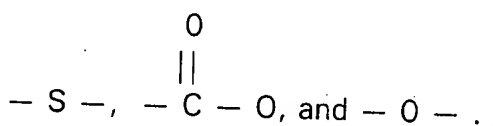
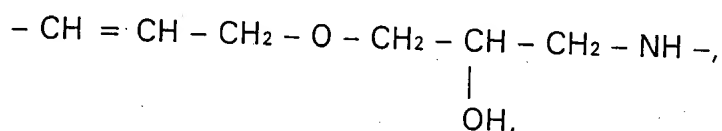
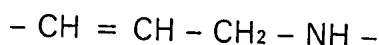
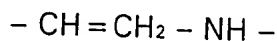


662. The oligo- or polydeoxyribonucleotide of claim 658, wherein said chemical linkage does not interfere substantially with the characteristic ability of Sig to form a detectable signal.

663. The oligo- or polydeoxyribonucleotide of claim 658, wherein said chemical linkage comprises a member selected from the group consisting of an olefinic bond at the α -position relative to the point of attachment to the nucleotide, a $-\text{CH}_2\text{NH}-$ moiety, or both.

664. The oligo- or polydeoxyribonucleotide of claim 658, wherein said chemical linkage comprises an allylamine group.

665. The oligo- or polydeoxyribonucleotide of claim 658, wherein said chemical linkage comprises or includes an olefinic bond at the α -position relative to the point of attachment to the nucleotide, or any of the moieties:



666. The oligo- or polydeoxyribonucleotide of claim 658, wherein said chemical linkage of Sig includes a glycosidic linkage moiety.

667. The oligo- or polydeoxyribonucleotide of claim 658, wherein said PM is monophosphate, a diphosphate or a triphosphate and said Sig moiety is covalently attached to said PM through a phosphorus atom or phosphate oxygen.

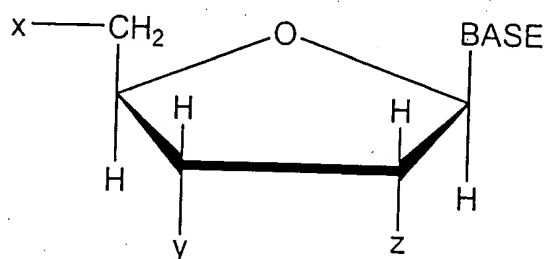
668. The oligo- or polydeoxyribonucleotide of claim 658, wherein said electron dense component comprises ferritin.

669. The oligo- or polydeoxyribonucleotide of claim 658, wherein said magnetic component comprises magnetic oxide.

670. The oligo- or polydeoxyribonucleotide of claim 658, wherein said magnetic oxide comprises ferric oxide.

671. The oligo- or polydeoxyribonucleotide of claim 658, wherein said metal-containing component is catalytic.
672. The oligo- or polydeoxyribonucleotide of claim 658, wherein said fluorescent component comprises a member selected from the group consisting of fluorescein, rhodamine and dansyl.
673. The oligo- or polydeoxyribonucleotide of claim 658, wherein said Sig moiety is attached to a terminal nucleotide in said oligo- or polydeoxyribonucleotide.
674. The oligo- or polydeoxyribonucleotide of claim 673, wherein the sugar moiety of said terminal nucleotide has a hydrogen atom at the 2' position thereof.
675. The oligo- or polydeoxyribonucleotide of claim 673, wherein the sugar moiety of said terminal nucleotide has oxygen atoms at each of the 2' and 3' positions thereof.
676. The oligo- or polydeoxyribonucleotide of claim 658, comprising at least one ribonucleotide.

677. An oligo- or polydeoxyribonucleotide which is complementary to a nucleic acid of interest or a portion thereof, said oligo- or polydeoxyribonucleotide comprising at least one modified nucleotide having the structural formula:



wherein BASE is a moiety selected from the group consisting of a pyrimidine, a purine and a deazapurine, or analog thereof, and wherein BASE is attached to the 1' position of the pentose ring from the N1 position when BASE is a pyrimidine or from the N9 position when BASE is a purine or a deazapurine;

wherein x is selected from the group consisting of H—, HO—, a mono-phosphate, a di-phosphate and a tri-phosphate;

wherein y is selected from the group consisting of H—, HO—, a mono-phosphate, a di-phosphate and a tri-phosphate;

wherein z is selected from the group consisting of H—, HO—, a mono-phosphate, a di-phosphate and a tri-phosphate; and

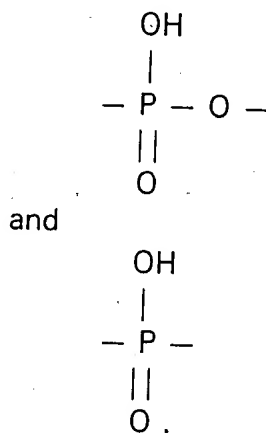
wherein Sig is covalently attached directly or through a chemical linkage to at least one phosphate selected from the group consisting of x, y, z, and a combination thereof, said Sig comprising a non-radioactive label moiety which can be directly or indirectly detected when so attached to said phosphate or when said modified nucleotide is incorporated into said oligo- or polydeoxyribonucleotide or when said oligo- or polydeoxyribonucleotide is hybridized to said complementary nucleic acid of interest or a portion thereof, wherein Sig comprises biotin, iminobiotin, an electron dense component, a magnetic component, a metal-

containing component, a fluorescent component, a chemiluminescent component, a chromogenic component or a combination of any of the foregoing.

678. The oligo- or polydeoxyribonucleotide of claim 677, wherein said Sig is or renders the nucleotide or the oligo- or polydeoxyribonucleotide self-signaling or self-indicating or self-detecting.

679. The oligo- or polydeoxyribonucleotide of claim 677, wherein said Sig moiety comprises at least three carbon atoms.

680. The oligo- or polydeoxyribonucleotide of claim 677, wherein said covalent attachment is selected from the group consisting of

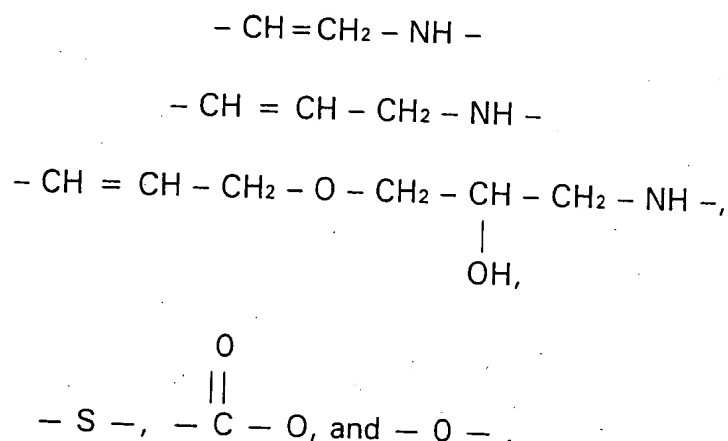


681. The oligo- or polydeoxyribonucleotide of claim 677, wherein said chemical linkage does not interfere substantially with the characteristic ability of Sig to form a detectable signal.

682. The oligo- or polydeoxyribonucleotide of claim 677, wherein said chemical linkage comprises a member selected from the group consisting of an olefinic bond at the α -position relative to the point of attachment to the nucleotide, a $-\text{CH}_2\text{NH}-$ moiety, or both.

683. The oligo- or polydeoxyribonucleotide of claim 677, wherein said chemical linkage comprises an allylamine group.

684. The oligo- or polydeoxyribonucleotide of claim 677, wherein said chemical linkage comprises or includes an olefinic bond at the α -position relative to the point of attachment to x, y or z, or any of the moieties:



685. The oligo- or polydeoxyribonucleotide of claim 677, wherein said chemical linkage of Sig includes a glycosidic linkage moiety.

686. The oligo- or polydeoxyribonucleotide of claim 677, wherein said x and y each comprise a member selected from the group consisting of a monophosphate, a diphosphate and a triphosphate and said Sig moiety is covalently attached to either or both of said x and y through a phosphorus atom or phosphate oxygen.

687. The oligo- or polydeoxyribonucleotide of claim 677, wherein said electron dense component comprises ferritin.

688. The oligo- or polydeoxyribonucleotide of claim 677, wherein said magnetic component comprises magnetic oxide.

689. The oligo- or polydeoxyribonucleotide of claim 688, wherein said magnetic oxide comprises ferric oxide.

690. The oligo- or polydeoxyribonucleotide of claim 677, wherein said metal-containing component is catalytic.

691. The oligo- or polydeoxyribonucleotide of claim 677, wherein said fluorescent component comprises a member selected from the group consisting of fluorescein, rhodamine and dansyl.

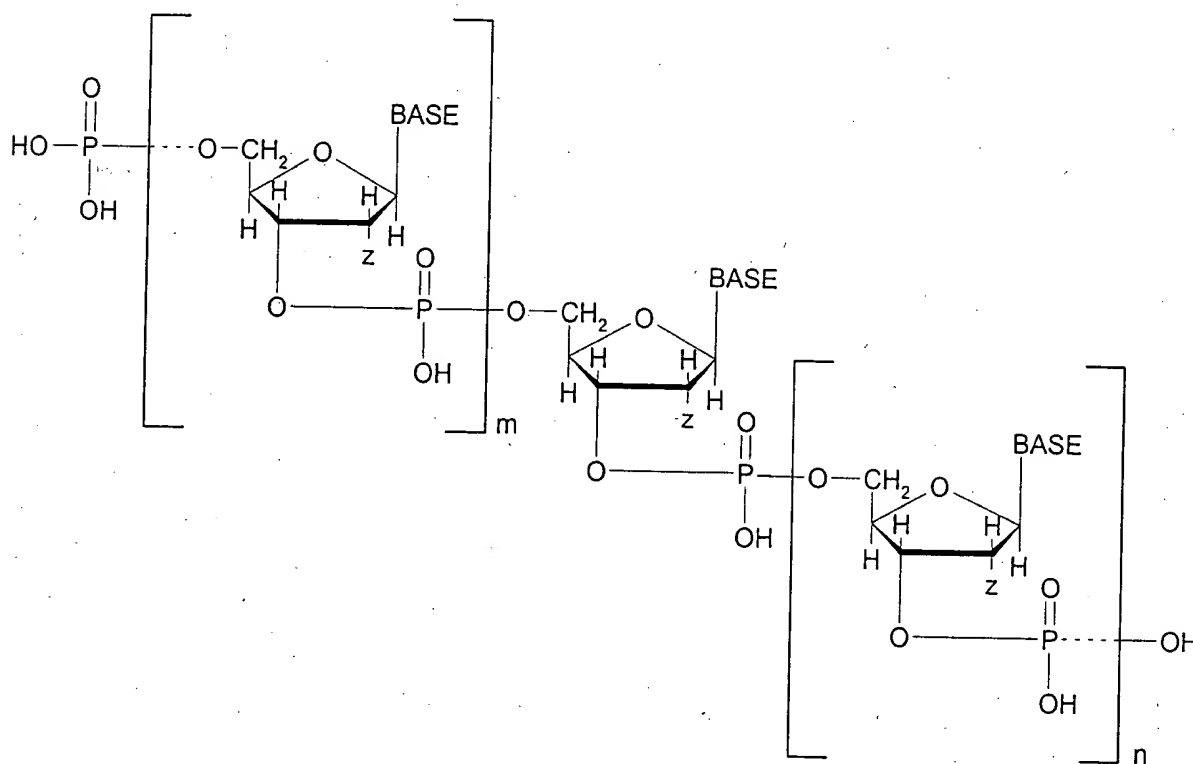
692. The oligo- or polydeoxyribonucleotide of claim 677, wherein said Sig moiety is attached to a terminal nucleotide in said oligo- or polydeoxyribonucleotide.

693. The oligo- or polydeoxyribonucleotide of claim 692, wherein z of said terminal nucleotide comprises a hydrogen atom at the 2' position thereof.

694. The oligo- or polydeoxyribonucleotide of claim 692, wherein both y and z of said terminal nucleotide comprise an oxygen atom at each of the 3' and 2' positions thereof, respectively.

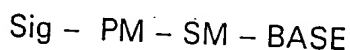
695. The oligo- or polydeoxyribonucleotide of claim 677, comprising at least one ribonucleotide.

696. The oligo- or polydexoyribonucleotide of claim 677, having the structural formula:



wherein m and n represent integers from 0 up to about 100,000, and wherein said Sig moiety is attached to at least one of the phosphate moieties in said structural formula.

697. An oligo- or polynucleotide which is complementary to a nucleic acid of interest or a portion thereof, said oligo- or polynucleotide comprising at least one modified nucleotide having the formula

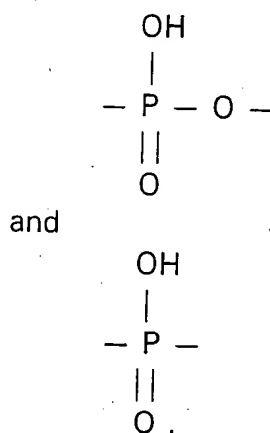


wherein PM is a phosphate moiety, SM is a sugar moiety and BASE is a moiety selected from the group consisting of a pyrimidine, a purine and a deazapurine, or analog thereof, said PM being attached to SM, said BASE being attached to SM, and Sig being covalently attached to PM directly or via a chemical linkage, said Sig comprising a non-radioactive label moiety which can be directly or indirectly detected when attached to PM or when said modified nucleotide is incorporated into said oligo- or polynucleotide, or when said oligo- or polynucleotide is hybridized to said complementary nucleic acid of interest or a portion thereof, provided that when said oligo- or polynucleotide is an oligoribonucleotide or a polyribonucleotide, and when Sig is attached through a chemical linkage to a terminal PM at the 3' position of a terminal ribonucleotide, said chemical linkage is not obtained through a 2',3' vicinal oxidation of a 3' terminal ribonucleotide previously attached to said oligoribonucleotide or polyribonucleotide, wherein Sig comprises biotin, iminobiotin, an electron dense component, a magnetic component, a metal-containing component, a fluorescent component, a chemiluminescent component, a chromogenic component or a combination of any of the foregoing.

698. The oligo- or polynucleotide of claim 697, wherein said Sig is or renders the nucleotide or the oligo- or polynucleotide self-signaling or self-indicating or self-detecting.

699. The oligo- or polynucleotide of claim 697, wherein said Sig moiety comprises at least three carbon atoms.

700. The oligo- or polynucleotide of claim 697, wherein said covalent attachment is selected from the group consisting of

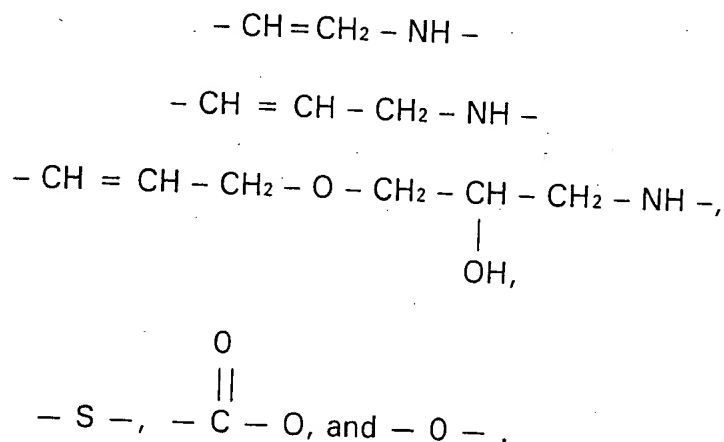


701. The oligo- or polynucleotide of claim 697, wherein said chemical linkage does not interfere substantially with the characteristic ability of Sig to form a detectable signal.

702. The oligo- or polynucleotide of claim 697, wherein said chemical linkage comprises a member selected from the group consisting of an olefinic bond at the α -position relative to the point of attachment to the nucleotide, a $-\text{CH}_2\text{NH}-$ moiety, or both.

703. The oligo- or polynucleotide of claim 697, wherein said chemical linkage comprises an allylamine group.

704. The oligo- or polynucleotide of claim 697, wherein said chemical linkage comprises or includes an olefinic bond at the α -position relative to the point of attachment to the nucleotide, or any of the moieties:



705. The oligo- or polynucleotide of claim 697, wherein said chemical linkage of Sig includes a glycosidic linkage moiety.

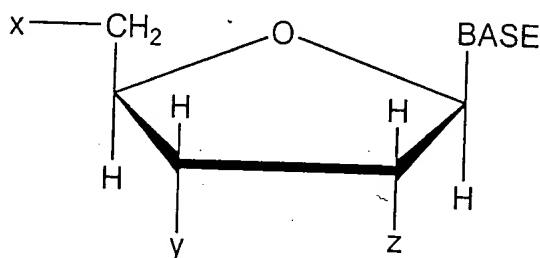
706. The oligo- or polynucleotide of claim 697, wherein said PM is a monophosphate, a diphosphate or a triphosphate and said Sig moiety is covalently attached to said PM through a phosphorus atom or a phosphate oxygen.

707. The oligo- or polynucleotide of claim 697, wherein said electron dense component comprises ferritin.

708. The oligo- or polynucleotide of claim 697, wherein said magnetic component comprises magnetic oxide.

709. The oligo- or polynucleotide of claim 708, wherein said magnetic oxide comprises ferric oxide.
710. The oligo- or polynucleotide of claim 697, wherein said metal-containing component is catalytic.
711. The oligo- or polynucleotide of claim 697, wherein said fluorescent component comprises a member selected from the group consisting of fluorescein, rhodamine and dansyl.
712. The oligo- or polynucleotide of claim 697, wherein said Sig moiety is attached to a terminal nucleotide in said oligo- or polynucleotide.
713. The oligo- or polynucleotide of claim 712, wherein the sugar moiety of said terminal nucleotide has a hydrogen atom at the 2' position thereof.
714. The oligo- or polynucleotide of claim 712, wherein the sugar moiety of said terminal nucleotide has an oxygen atom at each of the 2' and 3' positions thereof.
715. The oligo- or polynucleotide of claim 697, comprising at least one deoxyribonucleotide.

716. An oligo- or polynucleotide which is complementary to a nucleic acid of interest or a portion thereof, said oligo- or polynucleotide comprising at least one modified nucleotide having the structural formula:



wherein BASE is a moiety selected from the group consisting of a pyrimidine, a purine and a deazapurine, or analog thereof, and wherein BASE is attached to the 1' position of the pentose ring from the N1 position when BASE is a pyrimidine or from the N9 position when BASE is a purine or a deazapurine;

wherein x is selected from the group consisting of H—, HO—, a mono-phosphate, a di-phosphate and a tri-phosphate;

wherein y is selected from the group consisting of H—, HO—, a mono-phosphate, a di-phosphate and a tri-phosphate;

wherein z is selected from the group consisting of H—, HO—, a mono-phosphate, a di-phosphate and a tri-phosphate; and

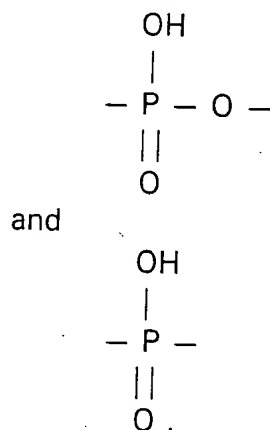
wherein Sig is covalently attached directly or through a chemical linkage to at least one phosphate selected from the group consisting of x, y and z, and a combination thereof, said Sig comprising a non-radioactive label moiety which can be directly or indirectly detected when so attached to said phosphate or when said modified nucleotide is incorporated into said oligo- or polynucleotide, or when said oligo- or polynucleotide is hybridized to said complementary nucleic acid of interest or a portion thereof, provided that when said oligo- or polynucleotide is an oligoribonucleotide or a polyribonucleotide and when Sig is attached through a

chemical linkage to a terminal PM at the 3' position of a terminal ribonucleotide, said chemical linkage is not obtained through a 2',3' vicinal oxidation of a 3' terminal ribonucleotide previously attached to said oligoribonucleotide or polyribonucleotide, wherein Sig comprises biotin, iminobiotin, an electron dense component, a magnetic component, a metal-containing component, a fluorescent component, a chemiluminescent component, a chromogenic component or a combination of any of the foregoing.

717. The oligo- or polynucleotide of claim 716, wherein said Sig is or renders the nucleotide or the oligo- or polynucleotide self-signaling or self-indicating or self-detecting.

718. The oligo- or polynucleotide of claim 716, wherein said Sig moiety comprises at least three carbon atoms.

719. The oligo- or polynucleotide of claim 716, wherein said covalent attachment is selected from the group consisting of

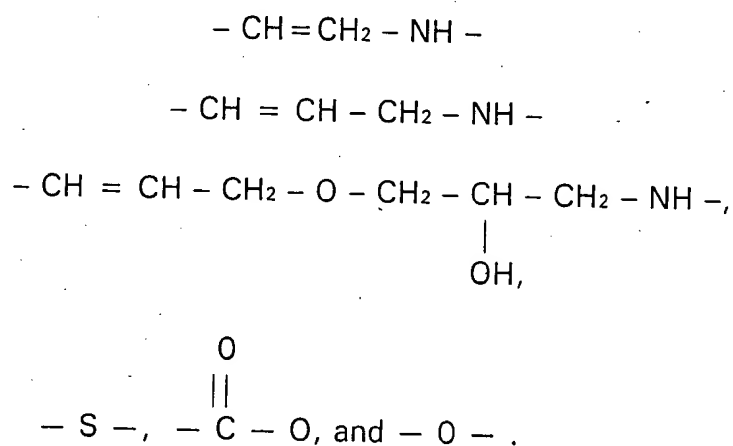


720. The oligo- or polynucleotide of claim 716, wherein said chemical linkage does not interfere substantially with the characteristic ability of Sig to form a detectable signal.

721. The oligo- or polynucleotide of claim 716, wherein said chemical linkage comprises a member selected from the group consisting of an olefinic bond at the α -position relative to the point of attachment to the nucleotide, a $-\text{CH}_2\text{NH}-$ moiety, or both.

722. The oligo- or polynucleotide of claim 716, wherein said chemical linkage comprises an allylamine group.

723. The oligo- or polynucleotide of claim 716, wherein said chemical linkage comprises or includes an olefinic bond at the α -position relative to x, y or z, or any of the moieties:



724. The oligo- or polynucleotide of claim 716, wherein said chemical linkage of Sig includes a glycosidic linkage moiety.

725. The oligo- or polynucleotide of claim 716, wherein said x and y each comprise a member selected from the group consisting of a monophosphate, a diphosphate and a triphosphate and Sig moiety is covalently attached to either or both of said x and y through a phosphorus atom or a phosphate oxygen.

726. The oligo- or polynucleotide of claim 716, wherein said electron dense component comprises ferritin.

727. The oligo- or polynucleotide of claim 716, wherein said magnetic component comprises magnetic oxide.

728. The oligo- or polynucleotide of claim 727, wherein said magnetic oxide comprises ferric oxide.

729. The oligo- or polynucleotide of claim 716, wherein said metal-containing component is catalytic.

730. The oligo- or polynucleotide of claim 716, wherein said fluorescent component comprises a member selected from the group consisting of fluorescein, rhodamine and dansyl.

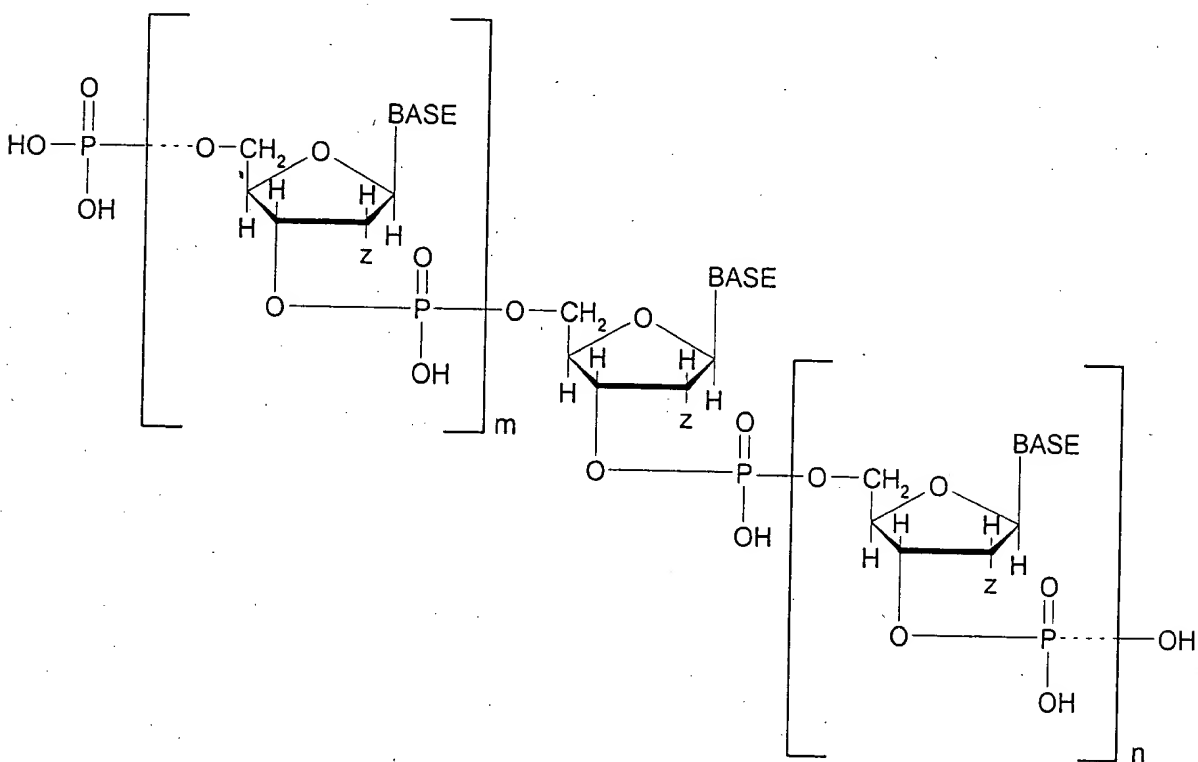
731. The oligo- or polynucleotide of claim 716, wherein said Sig moiety is attached to a terminal nucleotide in said oligo- or polynucleotide.

732. The oligo- or polynucleotide of claim 731, wherein z of said terminal nucleotide comprises a hydrogen atom at the 2' position thereof.

733. The oligo- or polynucleotide of claim 731, wherein both y and z of said terminal nucleotide comprise an oxygen atom at each of the 3' and 2' positions thereof, respectively.

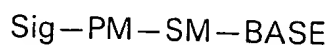
734. The oligo- or polynucleotide of claim 716, comprising at least one deoxyribonucleotide.

735. The oligo- or polynucleotide of claim 716, having the structural formula:



wherein m and n represent integers from 0 up to about 100,000, and wherein said Sig moiety is attached to at least one of the phosphate moieties in said structural formula.

736. An oligo- or polydeoxyribonucleotide which is complementary to a nucleic acid of interest or a portion thereof, said oligo- or polydeoxyribonucleotide comprising at least one modified nucleotide having the formula

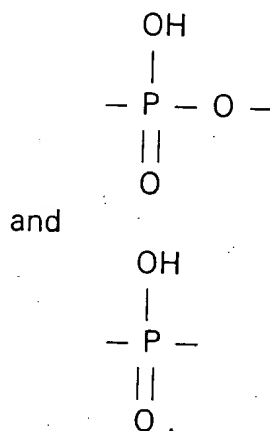


wherein PM is a phosphate moiety, SM is a sugar moiety and BASE is a base moiety selected from the group consisting of a pyrimidine, a purine and a deazapurine, or analog thereof, said PM being attached to SM, said BASE being attached to SM, and Sig being covalently attached to PM through a chemical linkage comprising a polypeptide or a protein, and said Sig comprising a non-radioactive label moiety which can be directly detected when indirectly attached to PM through said polypeptide or protein chemical linkage or when said modified nucleotide is incorporated into said oligo- or polydeoxyribonucleotide or when said oligo- or polydeoxyribonucleotide is hybridized to said complementary nucleic acid of interest or a portion thereof.

737. The oligo- or polydeoxyribonucleotide of claim 736, wherein said Sig is or renders the nucleotide or the oligo- or polydeoxyribonucleotide self-signaling or self-indicating or self-detecting.

738. The oligo- or polydeoxyribonucleotide of claim 736, wherein said Sig moiety comprises at least three carbon atoms.

739. The oligo- or polydeoxyribonucleotide of claim 736, wherein said covalent attachment is selected from the group consisting of



740. The oligo- or polydeoxyribonucleotide of claim 736, wherein said polypeptide or protein chemical linkage does not interfere substantially with the characteristic ability of Sig to form a detectable signal.

741. The oligo- or polydeoxyribonucleotide of claim 736, wherein said PM is monophosphate, a diphosphate or a triphosphate and said Sig moiety is covalently attached via said polypeptide or protein chemical linkage to said PM through a phosphorus atom or phosphate oxygen.

742. The oligo- or polydeoxyribonucleotide of claim 736, wherein Sig comprises a component selected from the group consisting of biotin, iminobiotin, an electron dense component, a magnetic component, a metal-containing component, a fluorescent component, a chemiluminescent component, a chromogenic component or a combination of any of the foregoing.

743. The oligo- or polydeoxyribonucleotide of claim 742, wherein said electron dense component comprises ferritin.

744. The oligo- or polydeoxyribonucleotide of claim 742, wherein said magnetic component comprises magnetic oxide.

745. The oligo- or polydeoxyribonucleotide of claim 744, wherein said magnetic oxide comprises ferric oxide.

746. The oligo- or polydeoxyribonucleotide of claim 742, wherein said metal-containing component is catalytic.

747. The oligo- or polydeoxyribonucleotide of claim 742, wherein said fluorescent component comprises a member selected from the group consisting of fluorescein, rhodamine and dansyl.

748. The oligo- or polydeoxyribonucleotide of claim 736, wherein said oligo- or polydeoxyribonucleotide is terminally ligated or attached to said polypeptide or protein chemical linkage.

749. The oligo- or polydeoxyribonucleotide of claim 736, wherein said polypeptide comprises polylysine.

750. The oligo- or polydeoxyribonucleotide of claim 736, wherein said polypeptide is selected from the group consisting of avidin, streptavidin and anti-hapten immunoglobulin.

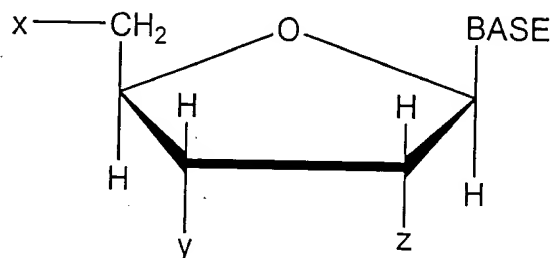
751. The oligo- or polydeoxyribonucleotide of claim 736, wherein said Sig moiety is attached via said polypeptide or protein chemical linkage to a phosphate moiety in a terminal nucleotide in said oligo- or polydeoxyribonucleotide.

752. The oligo- or polydeoxyribonucleotide of claim 751, wherein the sugar moiety of said terminal nucleotide has a hydrogen atom at the 2' position thereof.

753. The oligo- or polydeoxyribonucleotide of claim 751, wherein the sugar moiety of said terminal nucleotide has oxygen atoms at each of the 2' and 3' positions thereof.

754. The oligo- or polydeoxyribonucleotide of claim 736, comprising at least one ribonucleotide.

755. An oligo- or polydeoxyribonucleotide which is complementary to a nucleic acid of interest or a portion thereof, said oligo- or polydeoxyribonucleotide comprising at least one modified nucleotide having the structural formula:



wherein BASE is a moiety selected from the group consisting of a pyrimidine, a purine and a deazapurine, or analog thereof, and wherein BASE is attached to the 1' position of the pentose ring from the N1 position when BASE is a pyrimidine or from the N9 position when BASE is a purine or a deazapurine;

wherein x is selected from the group consisting of H—, HO—, a mono-phosphate, a di-phosphate and a tri-phosphate;

wherein y is selected from the group consisting of H—, HO—, a mono-phosphate, a di-phosphate and a tri-phosphate;

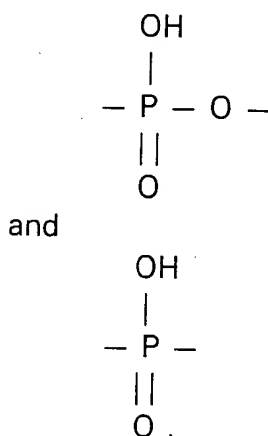
wherein z is selected from the group consisting of H—, HO—, a mono-phosphate, a di-phosphate and a tri-phosphate; and

wherein Sig is covalently attached through a chemical linkage to at least one phosphate selected from the group consisting of x, y, z, and a combination thereof, said chemical linkage comprising a polypeptide or a protein, and said Sig comprising a non-radioactive label moiety which can be directly or indirectly detected when attached to said phosphate via said polypeptide or protein chemical linkage or when said modified nucleotide is incorporated into said oligo- or polydeoxynucleotide or when said oligo- or polydeoxynucleotide is hybridized to said complementary nucleic acid of interest or a portion thereof.

756. The oligo- or polydeoxyribonucleotide of claim 755, wherein said Sig is or renders the modified nucleotide or the oligo- or polydeoxyribonucleotide self-signaling or self-indicating or self-detecting.

757. The oligo- or polydeoxyribonucleotide of claim 755, wherein said Sig moiety comprises at least three carbon atoms.

758. The oligo- or polydeoxyribonucleotide of claim 755, wherein said covalent attachment is selected from the group consisting of



759. The oligo- or polydeoxyribonucleotide of claim 755, wherein said polypeptide or protein chemical linkage does not interfere substantially with the characteristic ability of Sig to form a detectable signal.

760. The oligo- or polydeoxyribonucleotide of claim 755, wherein said x and y each comprise a member selected from the group consisting of a monophosphate, a diphosphate and a triphosphate and said Sig moiety is covalently attached via said polypeptide or protein chemical linkage to either or both of said x and y a phosphorus atom or phosphate oxygen.

761. The oligo- or polydeoxyribonucleotide of claim 755, wherein Sig comprises a component selected from the group consisting of biotin, iminobiotin, an electron dense component, a magnetic component, a metal-containing component, a fluorescent component, a chemiluminescent component, a chromogenic component, or a combination of any of the foregoing.

762. The oligo- or polydeoxyribonucleotide of claim 761, wherein said electron dense component comprises ferritin.

763. The oligo- or polydeoxyribonucleotide of claim 761, wherein said magnetic component comprises magnetic oxide.

764. The oligo- or polydeoxyribonucleotide of claim 763, wherein said magnetic oxide comprises ferric oxide.

765. The oligo- or polydeoxyribonucleotide of claim 761, wherein said metal-containing component is catalytic.

766. The oligo- or polydeoxyribonucleotide of claim 761, wherein said fluorescent component comprises a member selected from the group consisting of fluorescein, rhodamine and dansyl.

767. The oligo- or polydeoxyribonucleotide of claim 755, wherein said oligo- or polydeoxyribonucleotide is terminally ligated or attached to said polypeptide or protein chemical linkage.

768. The composition of claim 755, wherein said polypeptide comprises polylysine.

769. The composition of claim 755, wherein said polypeptide is selected from the group consisting of avidin, streptavidin and anti-hapten immunoglobulin.

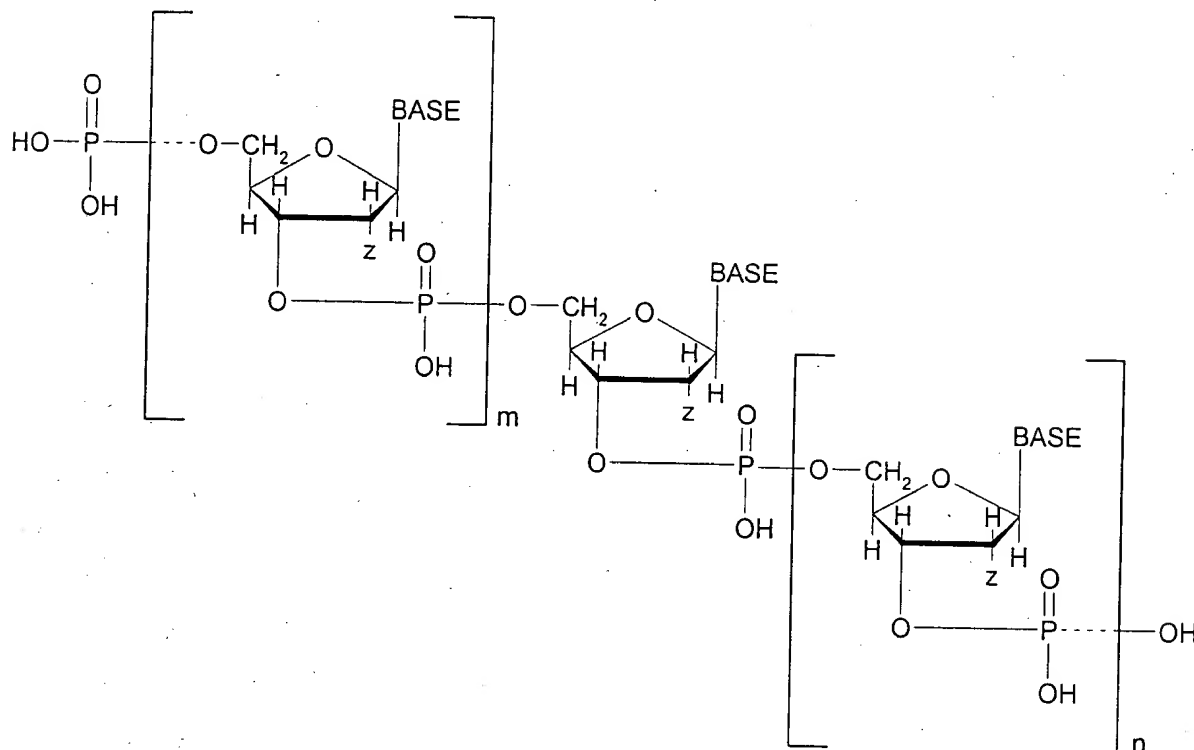
770. The oligo- or polydeoxyribonucleotide of claim 755, wherein said Sig moiety is attached via said polypeptide or protein chemical linkage to a terminal nucleotide in said oligo- or polydeoxyribonucleotide.

771. The oligo- or polydeoxyribonucleotide of claim 770, wherein z of said terminal nucleotide comprises a hydrogen atom at the 2' position thereof.

772. The oligo- or polydeoxyribonucleotide of claim 770, wherein both y and z of said terminal nucleotide comprise an oxygen atom at each of the 3' and 2' positions thereof, respectively.

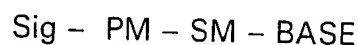
773. The oligo- or polydeoxyribonucleotide of claim 755, comprising at least one ribonucleotide.

774. The oligo- or polydeoxyribonucleotide of claim 755, having the structural formula:



wherein m and n represent integers from 0 up to about 100,000, and wherein said Sig moiety is attached to at least one of the phosphate moieties in said structural formula.

775. An oligo- or polynucleotide which is complementary to a nucleic acid of interest or a portion thereof, said oligo- or polynucleotide comprising at least one modified nucleotide having the formula

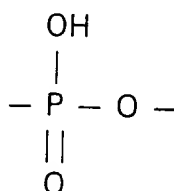


wherein PM is a phosphate moiety, SM is a sugar moiety and BASE is a moiety selected from the group consisting of a pyrimidine, a purine and a deazapurine, or analog thereof, said PM being attached to SM, said BASE being attached to SM, and Sig being covalently attached to PM via a chemical linkage comprising a polypeptide or a protein, said Sig comprising a non-radioactive label moiety which can be directly or indirectly detected when attached to PM via said polypeptide or protein chemical linkage or when said modified nucleotide is incorporated into said oligo- or polynucleotide, or when said oligo- or polynucleotide is hybridized to said complementary nucleic acid of interest or a portion thereof, provided that when said oligo- or polynucleotide is an oligoribonucleotide or a polyribonucleotide, and when Sig is attached through a chemical linkage to a terminal PM at the 3' position of a terminal ribonucleotide, said chemical linkage is not obtained through a 2',3' vicinal oxidation of a 3' terminal ribonucleotide previously attached to said oligoribonucleotide or polyribonucleotide.

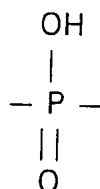
776. The oligo- or polynucleotide of claim 775, wherein said Sig is or renders the nucleotide or the oligo- or polynucleotide self-signaling or self-indicating or self-detecting.

777. The oligo- or polynucleotide of claim 775, wherein said Sig moiety comprises at least three carbon atoms.

778. The oligo- or polynucleotide of claim 775, wherein said covalent attachment is selected from the group consisting of



and



779. The oligo- or polynucleotide of claim 775, wherein said polypeptide or protein chemical linkage does not interfere substantially with the characteristic ability of Sig to form a detectable signal.

780. The oligo- or polynucleotide of claim 775, wherein said PM is a monophosphate, a diphosphate or a triphosphate and said Sig moiety is covalently attached via said polypeptide or protein chemical linkage to said PM through a phosphorus atom or a phosphate oxygen.

781. The oligo- or polynucleotide of claim 775, wherein Sig comprises a component selected from the group consisting of biotin, iminobiotin, an electron dense component, a magnetic component, a metal-containing component, a fluorescent component, a chemiluminescent component, a chromogenic component or a combination of any of the foregoing.

782. The oligo- or polynucleotide of claim 781, wherein said electron dense component comprises ferritin.

783. The oligo- or polynucleotide of claim 781, wherein said magnetic component comprises magnetic oxide.
784. The oligo- or polynucleotide of claim 783, wherein said magnetic oxide comprises ferric oxide.
785. The oligo- or polynucleotide of claim 781, wherein said metal-containing component is catalytic.
786. The oligo- or polynucleotide of claim 781, wherein said fluorescent component comprises a member selected from the group consisting of fluorescein, rhodamine and dansyl.
787. The oligo- or polynucleotide of claim 775, wherein said oligo- or polynucleotide is terminally ligated or attached to said polypeptide or protein chemical linkage.
788. The oligo- or polynucleotide of claim 775, wherein said polypeptide comprises polylysine.
789. The oligo- or polynucleotide of claim 775, wherein said polypeptide is selected from the group consisting of avidin, streptavidin and anti-hapten immunoglobulin.

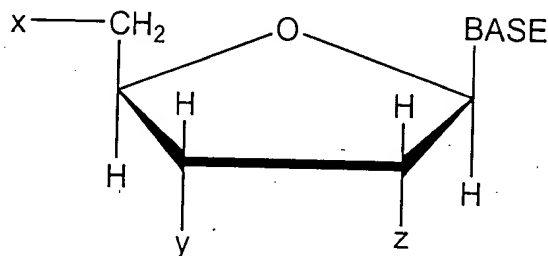
790. The oligo- or polynucleotide of claim 775, wherein said Sig moiety is attached via said polypeptide or protein chemical linkage to a terminal nucleotide in said oligo- or polynucleotide.

791. The oligo- or polynucleotide of claim 790, wherein the sugar moiety of said terminal nucleotide has a hydrogen atom at the 2' position thereof.

792. The oligo- or polynucleotide of claim 790, wherein the sugar moiety of said terminal nucleotide has an oxygen atom at each of the 2' and 3' positions thereof.

793. The oligo- or polynucleotide of claim 775, comprising at least one deoxyribonucleotide.

794. An oligo- or polynucleotide which is complementary to a nucleic acid of interest or a portion thereof, said oligo- or polynucleotide comprising at least one modified nucleotide having the structural formula:



wherein BASE is a moiety selected from the group consisting of a pyrimidine, a purine and a deazapurine, or analog thereof, and wherein BASE is attached to the 1' position of the pentose ring from the N1 position when BASE is a pyrimidine or from the N9 position when BASE is a purine or a deazapurine;

wherein x is selected from the group consisting of H—, HO—, a mono-phosphate, a di-phosphate and a tri-phosphate;

wherein y is selected from the group consisting of H—, HO—, a mono-phosphate, a di-phosphate and a tri-phosphate;

wherein z is selected from the group consisting of H—, HO—, a mono-phosphate, a di-phosphate and a tri-phosphate; and

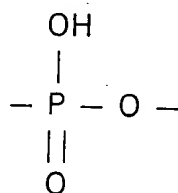
wherein Sig is covalently attached through a chemical linkage to at least one phosphate selected from the group consisting of x, y and z, and a combination thereof, said chemical linkage comprising a polypeptide or a protein, and said Sig comprising a non-radioactive label moiety which can be directly detected when attached to said phosphate via said polypeptide or protein chemical linkage or when said modified nucleotide is incorporated into said oligo- or polynucleotide, or when said oligo- or polynucleotide is hybridized to said complementary nucleic acid of interest or a portion thereof, provided that when said oligo- or polynucleotide is an oligoribonucleotide or a polyribonucleotide and when Sig is attached through a

chemical linkage to a terminal PM at the 3' position of a terminal ribonucleotide, said chemical linkage is not obtained through a 2',3' vicinal oxidation of a 3' terminal ribonucleotide previously attached to said oligoribonucleotide or polyribonucleotide.

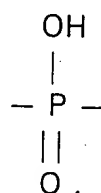
795. The oligo- or polynucleotide of claim 794, wherein said Sig is or renders the nucleotide or the oligo- or polynucleotide self-signaling or self-indicating or self-detecting.

796. The oligo- or polynucleotide of claim 794, wherein said Sig moiety comprises at least three carbon atoms.

797. The oligo- or polynucleotide of claim 794, wherein said covalent attachment is selected from the group consisting of



and



798. The oligo- or polynucleotide of claim 794, wherein said polypeptide or protein chemical linkage does not interfere substantially with the characteristic ability of Sig to form a detectable signal.

799. The oligo- or polynucleotide of claim 794, wherein said x and y each comprise a member selected from the group consisting of a monophosphate, a diphosphate and a triphosphate and Sig moiety is covalently attached to either or both of said x and y a phosphorus atom or a phosphate oxygen.

800. The oligo- or polynucleotide of claim 794, wherein Sig comprises a component selected from the group consisting of biotin, iminobiotin, an electron dense component, a magnetic component, a metal-containing component, a fluorescent component, a chemiluminescent component, a chromogenic component or a combination of any of the foregoing.

801. The oligo- or polynucleotide of claim 800, wherein said electron dense component comprises ferritin.

802. The oligo- or polynucleotide of claim 800, wherein said magnetic component comprises magnetic oxide.

803. The oligo- or polynucleotide of claim 802, wherein said magnetic oxide comprises ferric oxide.

804. The oligo- or polynucleotide of claim 800, wherein said metal-containing component is catalytic.

805. The oligo- or polynucleotide of claim 800, wherein said fluorescent component comprises a member selected from the group consisting of fluorescein, rhodamine and dansyl.

806. The oligo- or polynucleotide of claim 794, wherein said oligo- or polynucleotide is terminally ligated or attached to said polypeptide or protein chemical linkage.

807. The oligo- or polynucleotide of claim 794, wherein said polypeptide comprises polylysine.

808. The oligo- or polynucleotide of claim 794, wherein said polypeptide is selected from the group consisting of avidin, streptavidin and anti-hapten immunoglobulin.

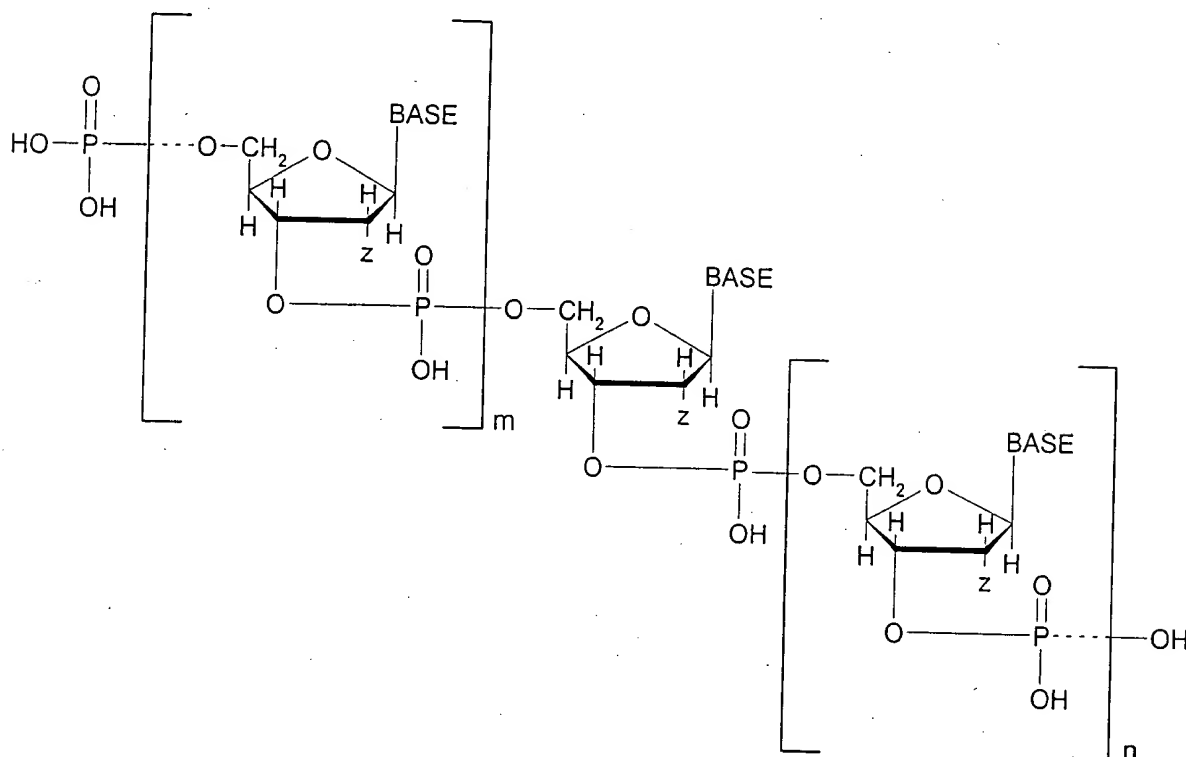
809. The oligo- or polynucleotide of claim 794, wherein said Sig moiety is attached via said polypeptide or protein chemical linkage to a terminal nucleotide in said oligo- or polynucleotide.

810. The oligo- or polynucleotide of claim 809, wherein z of said terminal nucleotide comprises a hydrogen atom at the 2' position thereof.

811. The oligo- or polynucleotide of claim 809, wherein both y and z of said terminal nucleotide comprise an oxygen atom at each of the 3' and 2' positions thereof, respectively.

812. The oligo- or polynucleotide of claim 794, comprising at least one deoxyribonucleotide.

813. The oligo- or polynucleotide of claim 794, having the structural formula:



wherein m and n represent integers from 0 up to about 100,000, and wherein said Sig moiety is attached to at least one of the phosphate moieties in said structural formula.

814. The oligo- or polydeoxyribonucleotide of claims 454 or 658, wherein said Sig is covalently attached to PM through a chemical linkage comprising a polypeptide or a protein.

815. The oligo- or polydeoxyribonucleotide of claim 814, wherein said polypeptide comprises polylysine.

816. The oligo- or polydeoxyribonucleotide of claim 814, wherein said polypeptide or protein is selected from the group consisting of avidin, streptavidin and anti-hapten immunoglobulin.

817. The oligo- or polydeoxyribonucleotide of claims 596 or 677, wherein said Sig is covalently attached to said at least one phosphate through a chemical linkage comprising a polypeptide or a protein.

818. The oligo- or polydeoxyribonucleotide of claim 817, wherein said polypeptide comprises polylysine.

819. The oligo- or polydeoxyribonucleotide of claim 817, wherein said polypeptide or protein is selected from the group consisting of avidin, streptavidin and anti-hapten immunoglobulin.

820. The oligo- or polynucleotide of claims 617 or 697, wherein said Sig is covalently attached to PM via a chemical linkage comprising a polypeptide or a protein.

821. The oligo- or polydeoxyribonucleotide of claim 820, wherein said polypeptide comprises polylysine.

822. The oligo- or polydeoxyribonucleotide of claim 820, wherein said polypeptide or protein is selected from the group consisting of avidin, streptavidin and anti-hapten immunoglobulin.

823. The oligo- or polynucleotide of claims 637 or 716, wherein said Sig is covalently attached to said at least one phosphate through a chemical linkage comprising a polypeptide or a protein.

824. The oligo- or polydeoxyribonucleotide of claim 823, wherein said polypeptide comprises polylysine.

825. The oligo- or polydeoxyribonucleotide of claim 824, wherein said polypeptide or protein is selected from the group consisting of avidin, streptavidin and anti-hapten immunoglobulin.

* * * * *

(b) Coenzyme A (Peak B, Fig. 3).—The ratio of phosphorus to adenosine was found to be 2.96 and the compound was chromatographically and electrophoretically identical with natural Coenzyme A. Degradation with crude rattlesnake venom gave adenosine-3',5'-diphosphate as the only detectable nucleotide. Enzymatically assayed as above, the compound gave a linear response of activity with concentration for 0.0041 and 0.0082 μ mole (optical density) and gave an activity of 139% in each case assuming a purity of 75% (by weight) for commercial Coenzyme A. Using the purified commercial product (see below) as the standard and assuming 100% activity on the basis of its adenosine content, the synthetic sample had 96% activity.

Anal. Calcd. for $C_{21}H_{37}N_7O_{16}P_2SLi_2 \cdot 6H_2O$: C, 28.41; H, 5.08; N, 10.98. Found (after drying at 100°): C, 28.52; H, 4.98; N, 9.87.

Purification of Commercial Coenzyme A.—The contents of a freshly opened 25 mg. bottle of "75%" Coenzyme A¹ were dissolved in water (3 ml.) and adjusted to pH 6.0 with ammonium hydroxide. 2-Mercaptoethanol (3 ml.) was added and the mixture stored at room temperature for 4 hr. after which time it was diluted with water (15 ml.) and applied directly to a 2 \times 22 cm. column of DEAE cellulose in the chloride form. After washing the column with water until no further ultraviolet-absorbing material was present in the washing, elution was commenced using a linear salt gradient. The mixing vessel contained 1.5 liters of 0.003 *N* hydrochloric acid and the reservoir contained 1.5 liters of 0.15 *N* lithium chloride in 0.003 *N* hydrochloric acid. Ten ml. fractions were collected at the rate of 1 ml. per minute. Three distinct peaks and two small ones were detected by ultraviolet absorption at 257 $m\mu$ (Fig. 4). Peak I had λ_{max} 239 $m\mu$

and a second small maximum at 283 $m\mu$ (ϵ 239/ ϵ 283 = 5.5 at pH 2.7) and was obviously not a nucleotide. Peak II contained at least two superimposed compounds one having λ_{max} 243 $m\mu$, and the other λ_{max} 255 $m\mu$. Peak III (273 optical density units at 257 $m\mu$, 18 μ mole) was reduced Coenzyme A. Peaks IV and V were too small for identification but, from its position, IV is probably oxidized Coenzyme A.

Peak III was adjusted to pH 4.0 with lithium hydroxide and worked up as described for the synthetic material to give 16 mg. of lithium salt which was chromatographically shown (Solvent I) to contain only reduced Coenzyme A and a little of the disulfide form. The material was somewhat hydrated; two preparations having equivalent weights of 960 and 1050 by ultraviolet absorption.

Assayed by the phosphotransacetylase method against the same "75%" standard as used for the synthetic material, it now showed 143% activity on the basis of its adenosine content.

Characterization of Pⁱ, Pⁱ Bis-(2' (or 3')-phosphoryl adenosine-5') Pyrophosphate (XXVI).—Incubation of the sulfur-free product from peak IV (Fig. 3) with crude rattlesnake venom rapidly gave adenosine-2'(3'),5'-diphosphate as the only phosphorus containing product. On incubation with purified prostatic phosphomonoesterase it was slowly (\sim 75% in 24 hr.) dephosphorylated to give initially Pⁱ-2'(3')-phosphoryl adenosine-5' Pⁱ-adenosine-5'-pyrophosphate (XXVI), with loss of one phosphomonoester group and subsequently di-adenosine-5'-pyrophosphate which were isolated in Solvents VI and I, respectively. The chromatographically isolated initial dephosphorylation product was rapidly degraded by crude venom, giving equal amounts of adenosine-2'(3'),5'-diphosphate, adenosine and inorganic phosphate. These results are all consistent with the structure assigned (XXVI) to this product.

(83) Pabst Laboratories, Milwaukee, Wis., Lot 413.

[CONTRIBUTION FROM THE CHEMISTRY DIVISION OF THE BRITISH COLUMBIA RESEARCH COUNCIL, VANCOUVER, B.C.]

Studies on Polynucleotides. VIII. Experiments on the Polymerization of Mononucleotides. Improved Preparation and Separation of Linear Thymidine Polynucleotides. Synthesis of Corresponding Members Terminated in Deoxycytidine Residues

By H. G. KHORANA¹ AND J. P. VIZSOLYI

RECEIVED JULY 18, 1960

Treatment of a molar anhydrous pyridine solution of a mixture of 3'-O-acetylthymidine-5' phosphate (25%) and thymidine-5' phosphate (75%) with dicyclohexylcarbodiimide at room temperature for six days gives linear thymidine polynucleotides as the major products. Members containing up to eleven units in a chain have been purified and characterized, smaller amounts of somewhat higher polynucleotides also being present in the polymerization mixtures. Procedures developed for the purification include chromatography of the total mixture on a DEAE-cellulose (carbonate) column using the volatile triethylammonium bicarbonate as the eluent and rechromatography of the major peaks under similar conditions. Polymerization of a mixture of N,3'-O-diacyldeoxycytidine-5' phosphate (25%) and thymidine-5' phosphate (75%) gives products from which thymidine polynucleotides bearing deoxycytidine residues at one end were isolated pure and characterized. The procedures developed for their purification involved, first, chromatography on DEAE-cellulose (carbonate) columns followed by rechromatography of the major peaks at acidic pH using the anion exchanger in the chloride form.

Introduction

The development of methods for the polymerization of mononucleotides and the separation and characterization of the resulting polymers forms a part of the program of synthetic work in the polynucleotide field which is in progress in this Laboratory.¹⁻⁶ The range of simple polymers

thus obtained offers obvious advantages for a variety of chemical, physico-chemical and enzymic studies in the nucleic acids field. The polymerizations of thymidine-5' phosphate and the isomeric 3'-phosphate by reaction with dicyclohexylcarbodiimide in anhydrous pyridine have previously been reported.^{7,8} While the extension of these initial studies to other mononucleotides and, indeed, in a number of directions is clearly desirable,⁹ many

(1) Paper VII, H. G. Khorana, *This Journal*, **81**, 4657 (1959).

(2) This work has been supported by grants from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service and the National Research Council of Canada, Ottawa.

(3) Institute for Enzyme Research, The University of Wisconsin, Madison 5, Wisconsin.

(4) G. M. Tener, P. T. Cilham, W. E. Razzell, A. F. Turner and H. G. Khorana, *Ann. N. Y. Acad. Sci.*, **81**, 757 (1959).

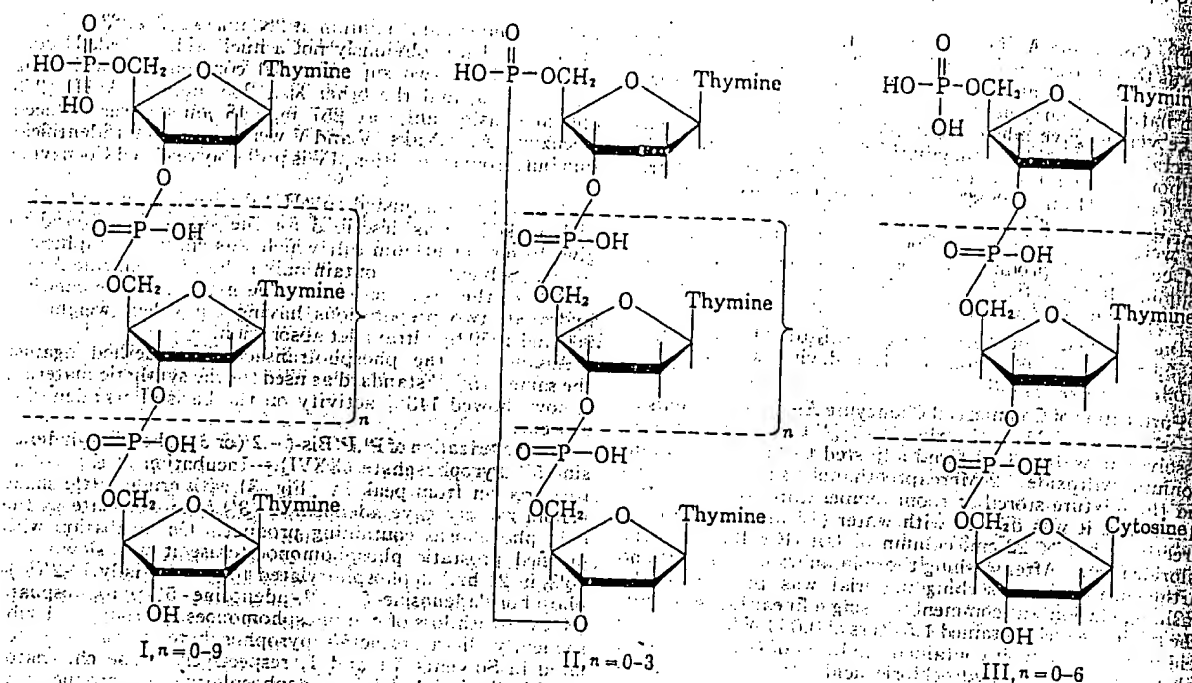
(5) H. G. Khorana, *J. Cellular Comp. Physiol.*, **54**, Suppl. 1, 5 (1959).

(6) H. G. Khorana, in E. Chargaff and J. N. Davidson, eds., "The Nucleic Acids," Vol. III, Academic Press, Inc., New York, N. Y., in press; H. G. Khorana, *Federation Proc.*, **50**, in press (1960).

(7) C. M. Tener, H. G. Khorana, R. Markham and E. H. Pol, *This Journal*, **80**, 8223 (1953).

(8) A. F. Turner and H. G. Khorana, *ibid.*, **81**, 4651 (1959).

(9) H. G. Khorana, A. F. Turner and J. P. Vizsolyi, *ibid.*, **83**, 686 (1961).



basic questions concerning chemical polymerization¹⁰ are being examined by further studying the relatively simple thymidine-5' phosphate itself. During all this work, marked improvements in the procedures, both for polymerization of this mononucleotide with a view to the preparation of linear polynucleotides (general structure, I) and for the separation of the polymers, have been effected. Because of the widespread interest in the thymidine polynucleotides of known size and structure, these procedures are described in this paper with special attention to experimental detail. The general principle used for favoring the formation of the linear polymers over the *cyclo*-oligonucleotides (general structure, II) has been applied to the preparation of thymidine polynucleotides bearing deoxycytidine residues at one end. The preparation and characterization of such compounds (general structure, III) are also described. The following paper⁹ records the synthesis of polynucleotides by polymerization of suitably protected deoxycytidine-5' phosphate.

In the previous work,^{7,8} a competing reaction in the linear polymerization was found to be the intramolecular phosphorylation of the 3'-hydroxyl group at one end of the chain by the activated 5'-phosphoryl group at the other end, resulting in the formation of the macrocyclic compounds of the type II. In fact, the cyclic dinucleotide (II, $n = 0$) accounted for 18-20% of the total nucleotidic material, and although the proportion of the higher cyclic members decreased with increase in chain length, it became insignificant only beyond the pentanucleotide level. An increase in the nucleotide concentration would be expected to favor linear polymerization (involving bimolecular reactions) and the present experiments have all been carried out using a much more concentrated (1 molar) solution of the nucleotide than that used previously.

The concentration (approximately 30% solution by weight of the nucleotide in pyridine) now used is as high as appears practical (see below). A technique which further reduces the extent of the cyclization reaction consists in the addition of some 3'-O-acetylthymidine-5' phosphate to thymidine-5' phosphate. The protected mononucleotide can only serve as the donor of an activated phosphoryl group and the chains formed with it as the terminal unit cannot undergo the intramolecular reaction. The addition of as much as 50% of 3'-O-acetylthymidine-5' phosphate completely inhibited the cyclization reaction but then, as expected, a large amount of mononucleotide was present in the final products.¹¹ In the polymerization experiments reported, 3'-O-acetylthymidine-5' phosphate and thymidine-5' phosphate were used in the ratio of 1:3. This ratio appears to represent a compromise, and although the amount of the cyclic dinucleotide formed is still high, the cyclic tri- and tetranucleotides are only minor products.

The above principle of polymerizing a nucleotide bearing the 3'-hydroxyl group in the presence of a second suitably protected mononucleotide should lead to an interesting general class of polymers, namely, 'homopolymers' terminated in a different nucleotide group. Compounds of this type are clearly useful for studies, such as the determination of the mode of action of phosphodiesterases¹² and nucleases.^{5,13} In the present work, homologous series of thymidine polynucleotides bearing deoxycytidine groups at one end (III) have been prepared.

New procedures for the isolation of pure homologous polynucleotides have been developed. A major technical advance is the use of the DEAE-cellulose columns⁷ in the bicarbonate form¹⁴ and of

(11) The collaboration of Dr. C. M. Tener in the early experiments is gratefully acknowledged.

(12) W. E. Razzell and H. G. Khorana, *J. Biol. Chem.*, **234**, 2114 (1959).

(13) H. G. Khorana, R. A. Smith and R. K. Rulph, in preparation.

(10) These include studies of the kinetics of polymerization and a comparative study of the efficiency of different chemical polymerizing agents such as "reactive" anhydrides.

(14) M. Staehelin, H. A. Sober and E. A. Peterson, *Arch. Biochem. Biophys.*, **85**, 239 (1959). These authors use ammonium carbonate

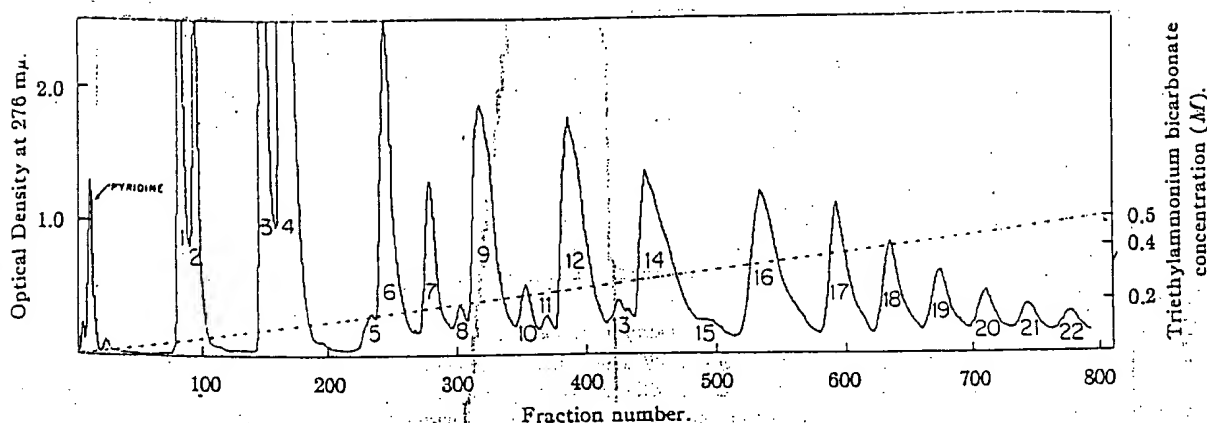


Fig. 1.—Chromatography of thymidine polynucleotides (total polymeric mixture) on DEAE-cellulose (bicarbonate) column. For details of procedure see text; for product distribution and identification, see Table I. Broken line shows triethylammonium bicarbonate gradient.

the volatile triethylammonium bicarbonate¹⁸ as the eluent. Procedures for rechromatography of the initially obtained peaks under altered conditions (of salt gradient or pH) have been devised for most of the polynucleotides described here. These procedures enable the isolation of pure compounds on a scale much larger than is conveniently possible by chromatography on paper sheets described earlier.^{7,8}

System of Abbreviations.—The basic system of abbreviations for polynucleotides used in this and the following paper⁹ as has been adopted by the *Journal of Biological Chemistry*.¹⁹ These abbreviations have been used widely by different workers in recent years and are very convenient. Thus the trinucleotide III ($n = 1$) is abbreviated to d-pTpTpC,¹⁹ the letter "d" designating deoxyribonucleoside series. In the present work dealing with rather large polymers derived from one kind of mononucleotide, it has been found necessary to develop the existing system of abbreviations further. Penta- and higher polynucleotides of the general structure I and III will be abbreviated to pT(pT), pT and pT(pT), pC respectively. Thus, the octanucleotide (III, $n = 6$) will be designated pT(pT), pC. As in the general formulae for full structures (I and III) the basic unit for the present abbreviations is a trinucleotide.¹⁹ The two end units of a trinucleotide chain, being different from each other, have to be retained and it is only the internal nucleoside-5' phosphoryl units which can be considered to repeat.

Thymidine Polynucleotides

The elution pattern obtained on initial chromatography of the polymeric mixture (corresponding to 1 mmole of the starting nucleotide) is shown in Fig.

for elution. Triethylamine bicarbonate¹⁸ is even more volatile and is used routinely in this Laboratory.

(16) J. Porath, *Nature*, **175**, 478 (1955).

(10) See under "Instructions to Authors" in current issues of the *Journal of Biological Chemistry*.

(17) (a) According to the nomenclature previously proposed,¹⁹ the trinucleotide would be named either 5-O-phosphorylthymidylyl-(3' → 5')-thymidylyl-(3' → 5') deoxycytidylyl-(5' → 3')-thymidylyl-(5' → 3')-thymidylic-(5') acid. (b) The significant shortening and convenience is effected only with the penta- and higher polynucleotides and therefore the abbreviations are introduced in this paper only from the pentanucleotide on.

(18) P. T. Gilham and H. G. Khorana, *This Journal*, **80**, 6212 (1953).

1. The manner of pooling the fractions and the distribution of the nucleotidic material in the different peaks are shown in Table I. The recovery as

TABLE I

CHROMATOGRAPHY OF THYMIDINE POLYNUCLEOTIDES. DISTRIBUTION OF NUCLEOTIDE MATERIAL IN DIFFERENT PEAKS OF FIGURE 1

Peak	Fractions pooled	Total nucleotide material ^a in peaks, %	Remarks, composition of the peak, etc.
1	78-87	3.63	Mainly N-pyridinium nucleotide compound
2	88-93	1.07	Discarded
3	94-102	2.53	Mainly thymidine 3',5'-cyclic phosphate
3a	146-156	6.50	Thymidine-5' phosphate
3b	157-162	1.45	Discarded
4	163-180	10.19	Mainly cyclic dinucleotide
5	225-238	0.76	Several unidentified components
6	239-265	5.83	Linear dinucleotide
7	272-295	3.45	Cyclic trinucleotide
8	296-308	0.87	Several unidentified components
9	310-345	9.24	Linear trinucleotide
10	346-363	1.42	Cyclic tetranucleotide
11	364-377	0.70	Mixture of unidentified components
12	378-415	8.97	Linear tetranucleotide
13	416-438	1.58	Cyclic pentanucleotide and unidentified compounds
14	439-485	7.80	Linear pentanucleotide
15	486-517	1.36	Not investigated
16	518-570	6.70	Linear hexanucleotide
16a	571-580	0.29	Not investigated
17	581-620	15.20	Linear heptanucleotide
18	621-660	4.12	Linear octanucleotide
19	661-697	3.19	Linear nonanucleotide
20	698-730	2.57	Linear decanucleotide
21	731-763	2.07	Linear undecanucleotide
22	764-793	1.52	Linear dodecanucleotide

1 M triethylammonium bicarbonate 4.71 Higher polymers

^a Total recovery of nucleotide material was 8,833 optical density units at 267 mμ. In view of the hypochromic effect in thymidine oligonucleotides,¹⁹ the recovery is concluded to be practically quantitative. ^b Percentage of the nucleotide material eluted after the tetranucleotide was 41.1%.

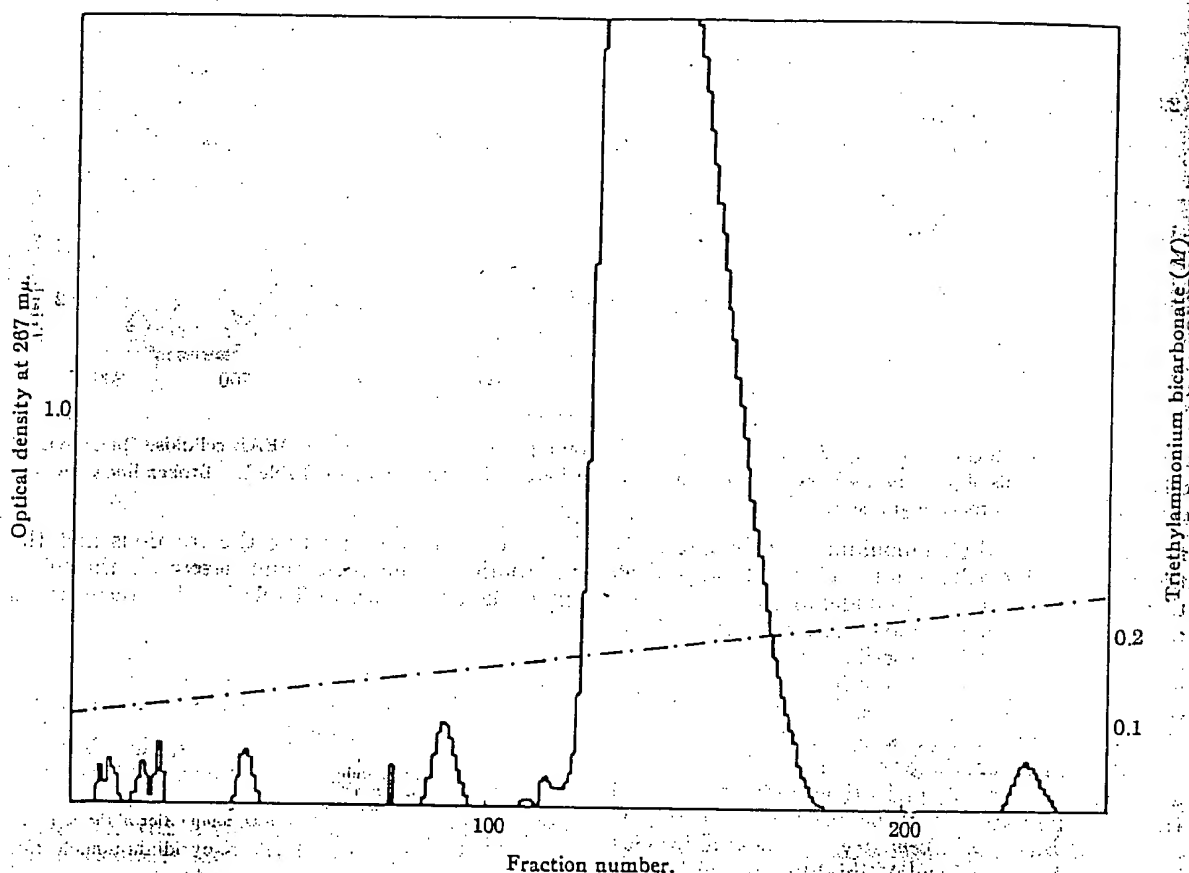


Fig. 2.—Rechromatography of the tetranucleotide peak (peak 12 of Fig. 1) on a DEAE-cellulose (bicarbonate) column (20 cm. \times 2 cm. dia.). The column was pre-equilibrated with 0.1 *M* triethylammonium bicarbonate buffer. Elution carried out using a linear gradient (broken line) of the same salt; the major peak is pure tetranucleotide.

judged by the ultraviolet absorption measurements was essentially quantitative.¹⁹ The resolution between the successive homologous polynucleotides was sustained as far as the elution was pursued by the gradient elution technique used. (In the previously published work,⁷ elution of pure peaks on a preparative scale was described only as far as the pentanucleotide.) The extent of polymerization achieved in these runs has been markedly higher than was obtained before.⁷ Thus 41% of the nucleotidic material appeared after the tetranucleotide (peak 12), about 5% of the total material being eluted after the dodecanucleotide peak (number 22) with 1 *M* triethylammonium bicarbonate. There was no sharp drop at any stage in the yield of the polymers, the amounts decreasing steadily with the increase in chain length after the linear tetranucleotide which accounted for 9.24% of the total nucleotidic material.

Linear Polynucleotides.—The linear polynucleotides were in peaks 6(dinucleotide), 9(trinucleotide), 12(tetranucleotide), 14(pentanucleotide), 16(hexanucleotide) and 17-22(heptanucleotide to dodecanucleotide).²⁰ For further purification, conditions were

found for rechromatography of each one of the polynucleotide peaks on DEAE-cellulose (carbonate form) columns. A shallower gradient was now used in each case and the various polynucleotides emerged from the columns at lower salt concentration than that at which they appeared in the initial gross chromatography. During rechromatography of the dinucleotide, it was found that satisfactory results were obtained by pre-equilibrating the column with the concentration of triethylammonium bicarbonate used initially in the mixing-vessel and this practice was followed for rechromatography of all of the higher polynucleotides. The conditions used for rechromatography and the yields of pure polynucleotides obtained are shown in Table II. The elution patterns obtained on rechromatography are illustrated with respect to the tetranucleotide (peak 12) and the decanucleotide (peak 20) peaks in Figs. 2 and 3, respectively. It was important to confirm that the increasing number of minor peaks²¹ obtained with increase in chain length were not due to any fault in the technique and therefore the main obtained on this scale have usually given more symmetrical peaks (see e.g. Fig. 4, below).

(21) As mentioned below, when the minor peaks (e.g., 5, 8 and 11 of Fig. 1) were examined by paper chromatography, each was found to contain several distinct bands. Since these minor peaks, which just preceded the linear polynucleotide peaks, were not separated beyond the heptanucleotide, they were, presumably, included in the main peaks and this phenomenon accounts for the increased number of small fore-peaks obtained upon rechromatography of the higher polynucleotide peaks.

(19) Some hypochromic effect in the synthetic polynucleotides is probable. Evidence for this at the dinucleotide level¹⁸ and in the higher thymidine oligonucleotides (unpublished experiments of C. M. Tener in this Laboratory) has been obtained.

(20) The skew nature of some of these peaks (tail end) does not indicate heterogeneity, as was shown by rechromatography of the individual peaks and subsequent tests of purity. The elution patterns

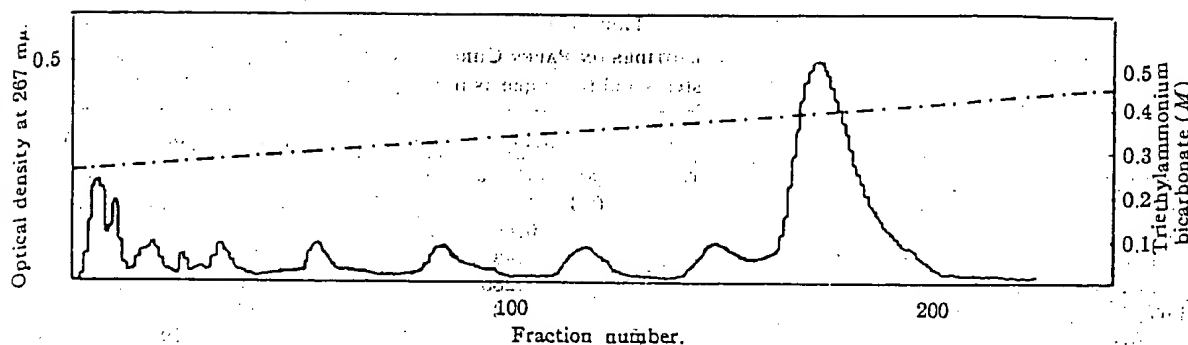


Fig. 3.—Rechromatography of the decanucleotide peak (peak 20 of Fig. 1) on a DEAE-cellulose (bicarbonate) column (20 cm. \times 2 cm. diameter). The column was pre-equilibrated with 0.25 *M* triethylammonium bicarbonate buffer. Elution with linear gradient of the same salt as shown by broken line; the peaks in the first ten fractions contain non-nucleotidic material.

peak of pure decanucleotide obtained in Fig. 3 was rechromatographed. A single sharp peak was again obtained (Fig. 4) in the expected region of salt concentration.

taining about 1 μ mole of thymidine. Single spots travelling faster than the starting materials were obtained.²³ A tracing of the chromatogram containing results with the octa-, nona- and deca-

TABLE II
RECHROMATOGRAPHY OF INDIVIDUAL PEAKS OF FIGURE 1 (THYMIDINE POLYNUCLEOTIDES) ON DEAE-CELLULOSE (CARBONATE) COLUMNS

(For details of procedure see text.)

Peak no. of Fig. 1	Poly-nucleotide	Conditions of rechromatography		Concentration of salt at mid-point of major peak	Yield pure oligonucleotide on rechromatography, %
		Mixing vessel ^a	Reservoir		
6	Di-	1 l. of 0.05 <i>M</i>	1 l. of 0.1 <i>M</i>	0.075	95
9	Tri-	1 l. of 0.075 <i>M</i>	1 l. of .15 <i>M</i>	.125	86
12	Tetra-	(1) 1 l. of .1 <i>M</i>	1 l. of .2 <i>M</i>	.180	84
		(2) 1/2 l. of .2 <i>M</i>	1/2 l. of .3 <i>M</i>		
14	Penta-	(1) 1 l. of .15 <i>M</i>	1 l. of .25 <i>M</i>	.230	87
		(2) 1/2 l. of .25 <i>M</i>	1/2 l. of .35 <i>M</i>		
16	Hexa-	2 l. of .2 <i>M</i>	2 l. of .4 <i>M</i>	.320	72
17	Hepta-	2 l. of .25 <i>M</i>	2 l. of .45 <i>M</i>	.340	69
18	Octa-	2 l. of .25 <i>M</i>	2 l. of .45 <i>M</i>	.350	70
19	Nona-	2 l. of .25 <i>M</i>	2 l. of .45 <i>M</i>	.360	52
20	Deca-	2 l. of .25 <i>M</i>	2 l. of .45 <i>M</i>	.380	56
21	Undeca-	2 l. of .25 <i>M</i>	2 l. of .45 <i>M</i>	.410	72

^a The column was pre-equilibrated with the concentration of triethylammonium bicarbonate (pH 7.5) used in the mixing vessel at the start of chromatography. ^b This is the % of the total ultraviolet absorbing material that was rechromatographed; the yields of each of the original peaks of Fig. 1 are given in Table I. ^c The actual recovery may be higher, since while investigating the appropriate conditions for rechromatography, the original peak had been put through columns twice. The yield recorded is that obtained in the final column and is based on the total optical density units in the original peak No. 16.

The purity of the oligonucleotides up to the pentanucleotide was checked by direct comparison with samples previously characterized,⁷ using extensive paper chromatography and paper electrophoresis. For the characterization of the higher members and for ascertaining their purity, chromatography on paper strips was performed over a period of three days to two weeks in several solvent systems. The pattern of the mobilities (see Table III for R_f 's) in all the solvent systems was consistent with their being a homologous series of compounds and single spots were uniformly obtained with all the polynucleotides. The most convincing proof of the homogeneity of the penta- to the deca-nucleotides was provided by dephosphorylation with the bacterial alkaline phosphomonoesterase²² followed by chromatography on paper using heavy spots con-

nucleotides is shown in Fig. 5. Finally, the degradation of the products lacking the terminal phosphomonoester groups by venom phosphodiesterase to thymidine-5' phosphate and thymidine, followed by estimation of the ratios of the two products gave results in good agreement with the size of the starting polynucleotides (Table IV).

Cyclic Oligonucleotides.—Peak 2 contained as the major constituent thymidine-3',5' cyclic phosphate,⁷ while peak 4 consisted mostly of cyclic

(23) Great emphasis is placed on this criterion of purity of the linear polynucleotides, since the general impurities that would be suspected from all the practical experience with the present method (see also the following paper²⁴) are of pyrophosphate type, the pyrophosphate bond being formed between the phosphomonoester groups of different oligonucleotides. These impurities could be eluted with or close to that polynucleotide bearing the 5'-phosphomonoester end group, which has the same net charge at pH 7.5. In the ammoniacal solvent systems, too, they may not be resolved from each other. Complete disappearance of the original spots (Fig. 5) on dephosphorylation constitutes the best means at the present time for showing freedom from the pyrophosphates.

(22) A. Caren and C. Leviathal, *Biochim. Biophys. Acta*, **38**, 470 (1960). We are very grateful for a sample of Dr. Caren's preparation of this very useful enzyme; the sample was kindly furnished to us by Dr. Leon A. Heppel.

TABLE III
R_f's OF POLYNUCLEOTIDES ON PAPER CHROMATOGRAMS
(Solvent systems and technique as in text)

Compound	A		B		C		D		E	
	R _f	R _f relative to pT ^b	R _f	R _f relative to pT	R _f	R _f relative to pT	R _f	R _f relative to pT	R _f	R _f relative to pT
pT	0.177	1	0.4	1	0.3	1	0.625	1	0.43	1
d-pC		0.86		1.19		0.66		1		1
d-pTpC		.47		0.89		.48		0.84		1
d-pTpTpC		.24		.56		.285		.69		1
d-pT(pT)pC		.094		.354		.13		.52		1
d-pT(pT)pC				0.71		0.60		.32		1
d-pT(pT)pC				.50		.33		0.79		1
d-pT(pT)pC				.37		.135		.58		1
d-pT(pT)pC				.24		.05		.42		1
d-pT(pT)pC				.16		.035		.276		1
pTpT		0.54		0.68		0.73		0.84		0.37
pTpTpT		.28		.49		.435		.63		.31
pTpTpTpT		.125		.285		.23		.44		.27
Thymidine 3',5' cyclic phosphate						1		1		.27
Cyclo-pTpT	0.47	2.65	0.52	1.3	0.58	1.93	0.67			
Cyclo-pTpTpT	0.24	1.4	0.30	0.75	0.386	1.29	.53			
Cyclo-pTpTpTpT	0.53		.51		0.79		.40			
pTpTpTpT	0.24		.35		0.43		.31			
pT(pT)pT			.695			0.57		0.72	0.22	
pT(pT)pT			.47			.29		.50	0.17	
pT(pT)pT			.325			.145		.36		0.34
pT(pT)pT			.25			.06		.25		.27
pT(pT)pT			.17			.029		.15		.20
pT(pT)pT										.14
pT(pT)pT										.09

* All compounds were spotted as ammonium salts; these were prepared by appropriate ion exchange techniques. * Chromatograms in these solvent systems, containing d-pTpTpTpC as the marker were run for 3 to 12 days. * Cyclo- before these oligonucleotides indicates that they are macrocyclic compounds of the general structure II ($n = 0-2$).

dinucleotide (II; $n = 0$). (Peak 3 was identified as thymidine-5' phosphate.) Cyclic tri- (II; $n = 1$) and tetra- (II; $n = 2$) nucleotides were present.

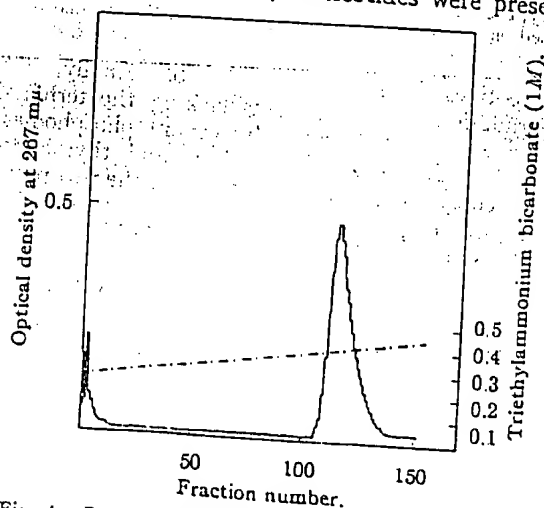


Fig. 4.—Rechromatography of the main decanucleotide peak of Fig. 3; conditions identical to those under Fig. 3. The peak in the first ten fractions contains non-nucleotidic material.

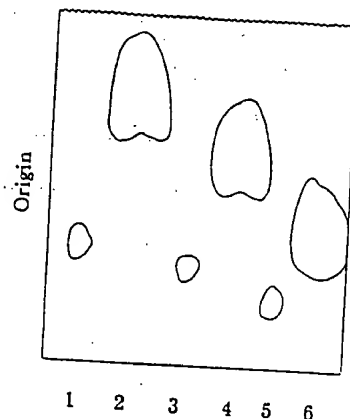


Fig. 5.—Paper chromatography of thymidine octa-, nona-, and decanucleotides before and after treatment with bacterial phosphomonoesterase. Solvent system, *n*-propyl alcohol-conc. ammonia-water (55-10-35), irrigation by the descending technique for 5½ days. Spots 1, 3 and 5, octa-, nona and decanucleotides before treatment with the phosphomonoesterase; spots 2, 4 and 6, products obtained after action of phosphomonoesterase from the octa-, nona- and decanucleotides, respectively. Each of the spots in the latter set contained a total of 1.6 μg.

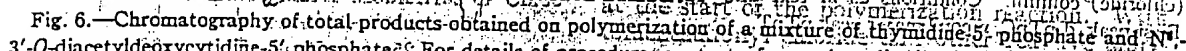


Fig. 6.—Chromatography of total products obtained on polymerization of a mixture of thymidine-5'-phosphate and N¹-3', 5'-bisphosphorylthymine at the start of the polymerization reaction. For detail, see text.

TABLE IV
RESULTS OF DEGRADATION OF THYMIDINE POLYNUCLEOTIDES LACKING TERMINAL PHOSPHOMONOESTER GROUPS BY VENOM PHOSPHOMONOSTERASE

(Details as in text)

	Density of optical density of spot at 267 mμ	Density of optical density of spot at 287 mμ	R ₀	Theor.
T(pT)pT	0.750	0.651	4.22	4.6
T(pT)pT	0.605	0.529	4.96	5.5
T(pT)pT	3.565	3.612	5.84	6.9
T(pT)pT	3.550	3.548	6.66	7.7
T(pT)pT	3.940	4.471	8.36	8.8
T(pT)pT	2.710	2.637	10.3	10.9

(24) There is seen on chromatograms in the isopropyl alcohol-ammonia-water solvent an additional spot with mobility similar to that of thymidine. The ultraviolet absorption characteristics (λ_{max} at 295 $m\mu$) show it to be non-nucleotide. The compound is encountered during acetylation of nucleotides and is apparently a product of reaction between acetic anhydride and pyridine.

General Remarks.—The method of polymerization is satisfactory for the preparation of poly- γ -thymidine nucleotides. However, the studies are required in order to conduct the chemical polymerization of γ -thymidine nucleotides will be required to determine the efficiency of the polymerization reaction. The all nucleotides in the mixture are heterogeneous numbers. One of the γ -thymidine nucleotides at the start of the polymerization reaction. Polymerization of a mixture of thymidine-5'-phosphate and N¹-phosphate, for product distribution and identification of the polymerization reaction. The compound isolated in the previous work would have the isomeric structure in which the pyridinium group is formed at the 5'-position and the phosphomonoester group is present at the 3'-hydroxyl group.

Thymidine, Polynucleotides Bearing Terminal Deoxycytidine Residues.—The separation of products obtained on polymerization of a mixture of N₃,5'-O-diacetyldeoxycytidine-5'-phosphate and thymidine-5'-phosphate is shown in Fig. 26. The fractions were pooled as shown in Table V, which also records the distribution of the nucleotidic material in the different peaks. The elution pattern is similar to that obtained above in Fig. 1, except that the polymerization did not go as far. The major peaks 8, 10, 12 and 14-19 contained the linear homologous polynucleotides and, as might have

(25) A possible mechanism for the formation of such compounds is the prior formation of an isourea ether by an addition reaction between 3'-hydroxyl group and dicyclohexylcarbodiimide and the subsequent attack of pyridine at C' with cleavage of the C-O bond. Such attack would be expected to occur from the back side and would result in inversion of configuration. In a model experiment O-methyl N,N-dicyclohexyl-isourea ether [J. C. Moffatt and H.G. Khorana, THIS JOURNAL, 79, 3741 (1957)] was rapidly cleaved by pyridine at room temperature to form N-methylpyridinium cation. However, all attempts to carry out addition reactions between the hydroxyl groups of thymidine and dicyclohexylcarbodiimide have failed. We are grateful to Drs. G. M. Tener and A. P. Turner for the experiments quoted here.

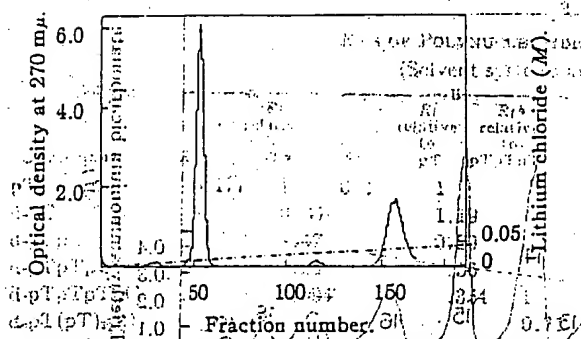


Fig. 7. Chromatography of the mixture of dinucleotides (d-pTpC and pTpT) (peak 8 of Fig. 6) on DEAE-cellulose (chloride) column. Conditions as in text and Table VI. First peak, d-pTpC; second major peak, pTpT.

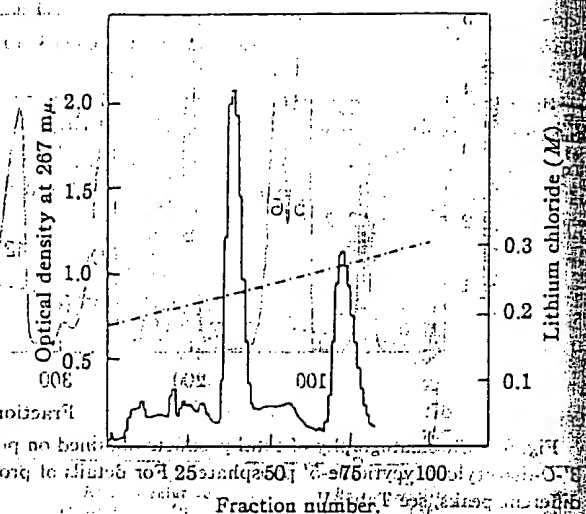


Fig. 8. Chromatography of the mixture of heptanucleotides (peak 16 of Fig. 6) on DEAE-cellulose (chloride) column. Conditions as in text and Table VI. First major peak, d-pTpTpC; second major peak, pTpTpTpT.

TABLE V
DISTRIBUTION OF NUCLEOTIDE MATERIAL IN DIFFERENT PEAKS OF FIGURE 6

Peak	Fractions	Total nucleic acid, %	Remarks
1	1-10	0.05	Discarded
2	11-15	0.05	Discarded
3	16-20	0.05	Discarded
4	21-25	0.05	Discarded
5	26-30	0.05	Discarded
6	31-35	0.05	Discarded
7	36-40	0.05	Discarded
8	41-45	0.05	Discarded
9	46-50	0.05	Discarded
10	51-55	0.05	Discarded
11	56-60	0.05	Discarded
12	61-65	0.05	Discarded
13	66-70	0.05	Discarded
14	71-75	0.05	Discarded
15	76-80	0.05	Discarded
16	81-85	0.05	Discarded
17	86-90	0.05	Discarded
18	91-95	0.05	Discarded
19	96-100	0.05	Discarded
20	101-105	0.05	Discarded
21	106-110	0.05	Discarded
22	111-115	0.05	Discarded
23	116-120	0.05	Discarded
24	121-125	0.05	Discarded
25	126-130	0.05	Discarded
26	131-135	0.05	Discarded
27	136-140	0.05	Discarded
28	141-145	0.05	Discarded
29	146-150	0.05	Discarded
30	151-155	0.05	Discarded
31	156-160	0.05	Discarded
32	161-165	0.05	Discarded
33	166-170	0.05	Discarded
34	171-175	0.05	Discarded
35	176-180	0.05	Discarded
36	181-185	0.05	Discarded
37	186-190	0.05	Discarded
38	191-195	0.05	Discarded
39	196-200	0.05	Discarded
40	201-205	0.05	Discarded
41	206-210	0.05	Discarded
42	211-215	0.05	Discarded
43	216-220	0.05	Discarded
44	221-225	0.05	Discarded
45	226-230	0.05	Discarded
46	231-235	0.05	Discarded
47	236-240	0.05	Discarded
48	241-245	0.05	Discarded
49	246-250	0.05	Discarded
50	251-255	0.05	Discarded
51	256-260	0.05	Discarded
52	261-265	0.05	Discarded
53	266-270	0.05	Discarded
54	271-275	0.05	Discarded
55	276-280	0.05	Discarded
56	281-285	0.05	Discarded
57	286-290	0.05	Discarded
58	291-295	0.05	Discarded
59	296-300	0.05	Discarded
60	301-305	0.05	Discarded
61	306-310	0.05	Discarded
62	311-315	0.05	Discarded
63	316-320	0.05	Discarded
64	321-325	0.05	Discarded
65	326-330	0.05	Discarded
66	331-335	0.05	Discarded
67	336-340	0.05	Discarded
68	341-345	0.05	Discarded
69	346-350	0.05	Discarded
70	351-355	0.05	Discarded
71	356-360	0.05	Discarded
72	361-365	0.05	Discarded
73	366-370	0.05	Discarded
74	371-375	0.05	Discarded
75	376-380	0.05	Discarded
76	381-385	0.05	Discarded
77	386-390	0.05	Discarded
78	391-395	0.05	Discarded
79	396-400	0.05	Discarded
80	401-405	0.05	Discarded
81	406-410	0.05	Discarded
82	411-415	0.05	Discarded
83	416-420	0.05	Discarded
84	421-425	0.05	Discarded
85	426-430	0.05	Discarded
86	431-435	0.05	Discarded
87	436-440	0.05	Discarded
88	441-445	0.05	Discarded
89	446-450	0.05	Discarded
90	451-455	0.05	Discarded
91	456-460	0.05	Discarded
92	461-465	0.05	Discarded
93	466-470	0.05	Discarded
94	471-475	0.05	Discarded
95	476-480	0.05	Discarded
96	481-485	0.05	Discarded
97	486-490	0.05	Discarded
98	491-495	0.05	Discarded
99	496-500	0.05	Discarded
100	501-505	0.05	Discarded
101	506-510	0.05	Discarded
102	511-515	0.05	Discarded
103	516-520	0.05	Discarded
104	521-525	0.05	Discarded
105	526-530	0.05	Discarded
106	531-535	0.05	Discarded
107	536-540	0.05	Discarded
108	541-545	0.05	Discarded
109	546-550	0.05	Discarded
110	551-555	0.05	Discarded
111	556-560	0.05	Discarded
112	561-565	0.05	Discarded
113	566-570	0.05	Discarded
114	571-575	0.05	Discarded
115	576-580	0.05	Discarded
116	581-585	0.05	Discarded
117	586-590	0.05	Discarded
118	591-595	0.05	Discarded
119	596-600	0.05	Discarded
120	601-605	0.05	Discarded
121	606-610	0.05	Discarded
122	611-615	0.05	Discarded
123	616-620	0.05	Discarded
124	621-625	0.05	Discarded
125	626-630	0.05	Discarded
126	631-635	0.05	Discarded
127	636-640	0.05	Discarded
128	641-645	0.05	Discarded
129	646-650	0.05	Discarded
130	651-655	0.05	Discarded
131	656-660	0.05	Discarded
132	661-665	0.05	Discarded
133	666-670	0.05	Discarded
134	671-675	0.05	Discarded
135	676-680	0.05	Discarded
136	681-685	0.05	Discarded
137	686-690	0.05	Discarded
138	691-695	0.05	Discarded
139	696-700	0.05	Discarded
140	701-705	0.05	Discarded
141	706-710	0.05	Discarded
142	711-715	0.05	Discarded
143	716-720	0.05	Discarded
144	721-725	0.05	Discarded
145	726-730	0.05	Discarded
146	731-735	0.05	Discarded
147	736-740	0.05	Discarded
148	741-745	0.05	Discarded
149	746-750	0.05	Discarded
150	751-755	0.05	Discarded
151	756-760	0.05	Discarded
152	761-765	0.05	Discarded
153	766-770	0.05	Discarded
154	771-775	0.05	Discarded
155	776-780	0.05	Discarded
156	781-785	0.05	Discarded
157	786-790	0.05	Discarded
158	791-795	0.05	Discarded
159	796-800	0.05	Discarded
160	801-805	0.05	Discarded
161	806-810	0.05	Discarded
162	811-815	0.05	Discarded
163	816-820	0.05	Discarded
164	821-825	0.05	Discarded
165	826-830	0.05	Discarded
166	831-835	0.05	Discarded
167	836-840	0.05	Discarded
168	841-845	0.05	Discarded
169	846-850	0.05	Discarded
170	851-855	0.05	Discarded
171	856-860	0.05	Discarded
172	861-865	0.05	Discarded
173	866-870	0.05	Discarded
174	871-875	0.05	Discarded
175	876-880	0.05	Discarded
176	881-885	0.05	Discarded
177	886-890	0.05	Discarded
178	891-895	0.05	Discarded
179	896-900	0.05	Discarded
180	901-905	0.05	Discarded
181	906-910	0.05	Discarded
182	911-915	0.05	Discarded
183	916-920	0.05	Discarded
184	921-925	0.05	Discarded
185	926-930	0.05	Discarded
186	931-935	0.05	Discarded
187	936-940	0.05	Discarded
188	941-945	0.05	Discarded
189	946-950	0.05	Discarded
190	951-955	0.05	Discarded
191	956-960	0.05	Discarded
192	961-965	0.05	Discarded
193	966-970	0.05	Discarded
194	971-975	0.05	Discarded
195	976-980	0.05	Discarded
196	981-985	0.05	Discarded
197	986-990	0.05	Discarded
198	991-995	0.05	Discarded
199	996-1000	0.05	Discarded

This is the total material eluted with 1 M triethylammonium bicarbonate, pH 7.5.

raphy of the total mixture was carried out under acidic (pH 2.7) conditions and there the separation of all of the expected small-sized compounds (d-pTpC, pTpT, d-pTpTpC, etc.) was satisfactory, but as the size increased, the compounds with approximately equal charge at the acidic pH (e.g. pTpTpT and d-pTpTpTpC) emerged together and further separation was carried out by paper chromatography. The procedures now preferred and described here

take advantage of the use of the volatile eluent for the initial gross separation (Fig. 6) into the pairs of each size and subsequent chromatography under acidic conditions of each one of the major linear polynucleotide peaks. Uniformly successful results were thus obtained, two examples of separation, namely, at the di- and heptanucleotide level, are shown in Figs. 7 and 8. Two major peaks were obtained in every case, the first one being, as expected, that of deoxycytidine-containing polynucleotide. The amount of the ultraviolet absorbing material separating from the major components increased with the increase in size, but the materials recovered from the main peaks were all pure (See

TABLE VI
SEPARATION OF THYMINE POLYNUCLEOTIDES FROM THE CORRESPONDING POLYNUCLEOTIDES CONTAINING TERMINAL DEOXYCYTIDINE BY RECHROMATOGRAPHY

Peak	Fractions	Total nucleic acid, %	Remarks
1	1-10	0.05	Discarded
2	11-15	0.05	Discarded
3	16-20	0.05	Discarded
4	21-25	0.05	Discarded
5	26-30	0.05	Discarded
6	31-35	0.05	Discarded
7	36-40	0.05	Discarded
8	41-45	0.05	Discarded
9	46-50	0.05	Discarded
10	51-55	0.05	Discarded
11	56-60	0.05	Discarded
12	61-65	0.05	Discarded
13	66-70	0.05	Discarded
14	71-75	0.05	Discarded
15	76-80	0.05	Discarded
16	81-85	0.05	Discarded
17	86-90	0.05	Discarded
18	91-95	0.05	Discarded
19	96-100	0.05	Discarded
20	101-105	0.05	Discarded
21	106-110	0.05	Discarded
22	111-115	0.05	Discarded
23	116-120	0.05	Discarded
24	121-125	0.05	Discarded
25	126-130	0.05	Discarded
26	131-135	0.05	Discarded
27	136-140	0.05	Discarded
28	141-145	0.05	Discarded
29	146-150	0.05	Discarded
30	151-155	0.05	Discarded
31	156-160	0.05	Discarded
32	161-165	0.05	Discarded
33	166-170	0.05	Discarded
34	171-175	0.05	Discarded
35	176-180	0.05	Discarded
36	181-185	0.05	Discarded
37	186-190	0.05	Discarded
38	191-195	0.05	Discarded
39	196-200	0.05	Discarded
40	201-205	0.05	Discarded
41	206-210	0.05	Discarded
42	211-215	0.05	Discarded
43	216-220	0.05	Discarded
44	221-225	0.05	Discarded
45	226-230	0.05	Discarded
46	231-235	0.05	Discarded
47	236-240	0.05	Discarded
48	241-245	0.05	Discarded
49	246-250	0.05	Discarded
50	251-255	0.05	Discarded
51	256-260	0.05	Discarded
52	261-265	0.05	Discarded
53	266-270	0.05	Discarded
54	271-275	0.05	Discarded
55	276-280	0.05	Discarded
56	281-285	0.05	Discarded
57	286-290	0.05	Discarded
58	291-295	0.05	Discarded
59	296-300	0.05	Discarded
60	301-305	0.05	Discarded
61	306-310	0.05	Discarded
62	311-315	0.05	Discarded
63	316-320	0.05	Discarded
64	321-325	0.05	Discarded
65	326-330	0.05	Discarded
66	331-335	0.05	Discarded
67	336-340	0.05	Discarded
68	341-345	0.05	Discarded
69	346-350	0.05	Discarded
70	351-355	0.05	Discarded
71	356-360	0.05	Discarded
72	361-365	0.05	Discarded
73	366-370	0.05	Discarded
74	371-375	0.05	Discarded
75	376-380	0.05	Discarded
76	381-385	0.05	Discarded
77	386-390	0.05	Discarded
78	391-395	0.05	Discarded
79	396-400	0.05	Discarded
80	401-405	0.05	Discarded
81	406-410	0.05	Discarded
82	411-415	0.05	Discarded
83	416-420	0.05	Discarded
84	421-425	0.05	Discarded
85	426-430	0.05	Discarded
86	431-435	0.05	Discarded
87	436-440	0.05	Discarded
88	441-445	0.05	Discarded
89	446-450	0.05	Discarded
90	451-455	0.05	Discarded
91	456-460	0.05	Discarded
92	461-465	0.05	Discarded
93	466-470	0.05	Discarded
94	471-475	0.05	Discarded
95	476-480	0.05	Discarded
96	481-485	0.05	Discarded
97	486-490	0.05	Discarded
98	491-495	0.05	Discarded
99	496-500	0.05	Discarded
100	501-505	0.05	Discarded
101	506-510	0.05	Discarded
102	511-515	0.05	Discarded
103	516-520	0.05	Discarded
104	521-525	0.05	Discarded
105	526-530	0.05	Discarded
106	531-535	0.05	Discarded
107	536-540	0.05	Discarded
108	541-545	0.05	Discarded
109	546-550	0.05	Discarded
110	551-555	0.05	Discarded
111	556-560	0.05	Discarded
112	561-565	0.05	Discarded
113	566-570	0.05	Discarded
114	571-575	0.05	Discarded
115	576-580	0.05	Discarded
116	581-585	0.05	Discarded
117	586-590	0.05	Discarded
118	591-595	0.05	Discarded
119	596-600	0.05	Discarded
120	601-605	0.05	Discarded
121	606-610	0.05	Discarded
122	611-615	0.05	Discarded
123	616-620	0.05	Discarded
124	621-625	0.05	Discarded
125	626-630	0.05	Discarded
126	631-635	0.05	Discarded
127	636-640	0.05	Discarded
128	641-645	0.05	Discarded
129	646-650	0.05	Discarded
130	651-655	0.05	Discarded
131	656-660	0.05	Discarded
132	661-665	0.05	Discarded
133	666-670	0.05	Discarded
134	671-675	0.05	Discarded
135	676-680	0.05	Discarded
136	681-685	0.05	Discarded
137	686-690	0.05	Discarded
138	691-695	0.05	Discarded
139	696-700	0.05	Discarded
140	701-705	0.05	Discarded
141	706-710	0.05	Discarded
142	711-715	0.05	Discarded
143	716-720	0.05	Discarded
144	721-725	0.05	Discarded
145	726-730	0.05	Discarded
146	731-735	0.05	Discarded
147	736-740	0.05	Discarded
148	741-745	0.05	Discarded
149	746-750	0.05	Discarded
150	751-755	0.05	Discarded
151	756-760	0.05	Discarded
152	761-765	0.05	Discarded
153	766-770	0.05	Discarded
154	771-775	0.05	Discarded
155	776-780	0.05	Discarded
156	781-785	0.05	Discarded
157	786-790	0.05	Discarded
158	791-795	0.05	Discarded
159	796-800	0.05	Discarded
160	801-805	0.05	Discarded
161	806-810	0.05	Discarded
162	811-815	0.05	Discarded
163	816-820	0.05	Discarded
164	821-825	0.05	Discarded
165	826-830	0.05	Discarded
166	831-835	0.05	Discarded
167	836-840	0.05	Discarded
168	841-845	0.05	Discarded
169	846-850	0.05	Discarded
170	851-855	0.05	Discarded
171	856-860	0.05	Discarded
172	861-865	0.05	Discarded
173	866-870	0.05	Discarded
174	871-875	0.05	Discarded
175	876-880	0.05	Discarded
176	881-885	0.05	Discarded
177	886-890	0.05	Discarded
178	891-895	0.05	Discarded
179	896-900	0.05	Discarded
180	901-905	0.05	Discarded
181	906-910	0.05	Discarded
182	911-915	0.05	Discarded
183	916-920	0.05	Discarded
184	921-925	0.05	Discarded
185	926-930	0.05	Discarded
186	931-935	0.05	Discarded
187	936-940	0.05	Discarded
188	941-945	0.05	Discarded
189	946-950	0.05	Discarded
190	951-955	0.05	Discarded
191	956-960	0.05	Discarded
192	961-965	0.05	Discarded
193	966-970	0.05	Discarded
194	971-975	0.05	Discarded
195	976-980	0.05	Discarded
196	981-985	0.05	Discarded
197	986-990	0.05	Discarded
198	991-995	0.05	Discarded
199	996-1000	0.05	Discarded
200	1001-1005	0.05	Discarded

below). The detailed conditions used for rechromatography and the proportions of the pure components containing thymidine only to their counterparts containing terminal deoxycytidine recovered at each level are listed in Table VI. The average ratio of the deoxycytidine member to the purely thymidine-containing member was 3:2.

The homogeneity of the polynucleotides bearing terminal deoxycytidine was established by extensive paper chromatography (Table III). Furthermore, paper electrophoresis at acidic pH proved especially useful in this series. The mobilities of the homologous members are listed in Table VII.

TABLE VII
RELATIVE PAPER-ELECTROPHORETIC MOBILITIES OF OLIGONUCLEOTIDES AT pH 3.5^a
(Details as in text)

Compound	Mobility relative to pTpTpC
pTpTpC	1.29
pTpTpTpC	1.47
pTpTpTpTpC	1.58
pT(pT)pT	1.64
d-pTpC	1.44
d-pTpTpC	1.63
d-pT(pT)pC	1.08
d-pT(pT)pC	1.15
d-pT(pT)pC	1.19

^a Electrophoresis run using 3" wide strips and a potential gradient of 15-16 volts/cm. Each run was performed for about 2 hr. in ammonium acetate (0.05 M) buffer. Mobilities are quoted relative to the reference compound run along with the oligonucleotides on the same strip.

(With the members containing thymidine only, the technique as hitherto used in this Laboratory was applicable only as far as the tetranucleotide—all the higher members having about the same mobility.) Degradation of the homologous polynucleotides by venom phosphodiesterase and determination of the ratios of the resulting deoxycytidine-5' phosphate and thymidine-5' phosphate gave excellent agreement with the values expected (Table VIII).

TABLE VIII
RESULTS OF DEGRADATION OF POLYNUCLEOTIDES TERMINATED IN DEOXYCYTIDINE BY VENOM PHOSPHODIESTERASE
(Details as in text)

Compound	Products of hydrolysis				Ratio pT/C Found	Theor.
	Optical density, ml. at 267 mμ	μmole ^a	Optical density, ml. at 280 mμ	μmole ^b		
d-pTpC	1.56	0.161	2.15	0.163	0.99	1
d-pTpTpC	4.10	.423	2.86	.2165	1.96	2
d-pTpTpTpC	4.30	.444	1.96	.148	3.00	3
d-pT(pT)pC	4.90	.505	1.64	.124	4.07	4
d-pT(pT)pC	3.28	.338	0.832	.067	5.04	5
d-pT(pT)pC	3.68	.379	0.840	.0636	5.96	6

^a Using a figure of 9,700 for ϵ_{max} at 267 mμ for thymidine-5' phosphate. ^b Using a figure of 13,200 for ϵ_{max} at 280 mμ in acid for deoxycytidine-5' phosphate.

The linear thymidine polynucleotides isolated were all pure when characterized as described in the preceding section. Other features of the elution diagram (Fig. 6) were similar to those of the diagram

in Fig. 1. Thus peaks 6, 9 and 11 mainly consisted of thymidine cyclic di-, tri- and tetra-nucleotides, respectively. The earlier peaks 2-4 had compositions similar to those described for Fig. 1 and peak 5 consisted of a mixture of deoxycytidine-5' phosphate and thymidine-5' phosphate.

General Remarks.—The method of polymerization as it stands is satisfactory for the preparation of linear polynucleotides containing up to about twelve units in a chain. However, further studies are required in order to induce the chemical polymerization to go much further. These studies will be concerned with a comparison of the efficiency of different polymerizing reagents. With dicyclohexylcarbodiimide the mixtures are heterogeneous at the start of the polymerization reaction. With toluenesulfonyl chloride and other reactive anhydrides²⁵ clear solutions result, and this factor may make kinetic studies of the polymerization reaction simpler. However, detailed analysis of products obtained by using reagents other than dicyclohexylcarbodiimide is necessary before further studies with these reagents can be undertaken. In the previous work⁷ the results obtained using dicyclohexylcarbodiimide were cleaner than when *p*-toluenesulfonylchloride was used.

Much effort continues to be expended in this Laboratory on the techniques for the separation of the synthetic polynucleotides. The procedures as now evolved are satisfactory for the purification of all of the products encountered, and it is hoped that the information that is being gained with the relatively simple polymeric mixtures will be of use in the formidable problems of separation of polynucleotides of natural origin.

Experimental
Preparation of N^{3'}-O-Diacetyldeoxycytidine-5' Phosphate and N^{3'}-O-Acetylthymidine-5' Phosphate.—Deoxycytidine-5' phosphate (free acid) and thymidine-5' phosphate used in the present work were commercial samples. They were checked carefully for their purity by (a) paper chromatography (solvents B and F; see below) on double acid washed paper strips, using at least 2 μmole of material for each spot; (b) paper electrophoresis; and (c) spectral characteristics. Paper chromatography in solvent B detects deoxyuridine-5' phosphate which may be present in thymidine-5' phosphate, while solvent F is suitable for detecting any ribonucleoside-5' phosphates.

Deoxycytidine-5' phosphate (0.5 mmole, 165 mg. of free acid) was dissolved in a mixture of 10 ml. of water and 1 ml. of pyridine and the solution lyophilized. The finely divided material thus obtained was suspended in 5 ml. of dry pyridine and 1.5 ml. of acetic anhydride added. The stoppered flask was kept in the dark at room temperature and shaken frequently. Clear solution resulted within a few hours. After a total of about 18 hr., water (20 ml.) was added to the essentially colorless solution in ice bath. The solution was kept at room temperature for about 1.5 hr. and then concentrated to a syrup *in vacuo* at low temperature (bath temperature below 20°) using a rotary evaporator. Water was added to the syrupy concentrate and the solution re-evaporated as above. The procedure was repeated twice when most of pyridinium acetate was removed. Finally an aqueous solution (about 60 ml.) of the product was lyophilized to give a fine white powder which was stored at 3° as a solution in pyridine and used directly. Paper chromatography in solvents C, D and G as well as paper electrophoresis at pH 3.5 showed the absence of any unacetylated material. A weak fast-travelling spot was frequently seen on chromatograms. This was evidently non-nucleotidic in character (λ_{max} , 295 mμ) and, as noted above, appears to

result from the reaction of acetic anhydride with pyridine alone. Another, very weak spot, possessing an acetylated deoxycytidine spectrum was also seen on chromatograms run in solvent D. This could be unhydrolyzed mixed anhydride between acetic acid and phosphate group of N,O-diacetyldeoxycytidine-5' phosphate. No attempt was made to remove this minor by-product.

The procedure for the acetylation of thymidine-5' phosphate was identical, except that the starting material, usually as ammonium salt, was first converted to the pyridinium salt by passage through a column of pyridinium Dowex-50 ion exchange resin, and the total effluent was evaporated, and the residue rendered anhydrous by repeated evaporation of its solution in pyridine. The reaction time usually given for this acetylation was around 7 hr. at room temperature in the dark, and it is not known if a dry atmosphere is required.

Polymerization of a Mixture of 3'-O-Acetylthymidine-5' Phosphate and Thymidine-5' Phosphate.—A mixture of pyridinium 3'-O-acetylthymidine-5' phosphate (as obtained by acetylating 1 mmole of thymidine-5' phosphate) and pyridinium thymidine-5' phosphate (3 mmole) was taken up in dry pyridine (10 ml.) and the solution evaporated to a gum *in vacuo* (oil pump) at low temperature. Dry air was admitted to the system, the residue redissolved in 10 ml. of dry pyridine and the solution re-evaporated as above. The whole procedure was repeated at least three times and the resulting anhydrous foam was taken up in 2 ml. of dry pyridine. To the clear solution was added under agitation from a pressure-equalizing flask a solution of dicyclohexylcarbodiimide (1.65 g., 8 mmole) in dry pyridine (2 ml.). The stoppered reaction vessel was vigorously shaken for some five minutes, during which time the initially separated mobile liquid turned into a gum. The two-phase mixture was shaken mechanically at room temperature in the dark for a total of six days. The gum progressively hardened and at the end the total reaction mixture turned into a solid mass because of the crystallization of additional amount of dicyclohexylurea. To the reaction mixture was then added rapidly, under shaking, an aqueous solution of sodium hydroxide (9 ml. of water + 6 ml. of 2 N sodium hydroxide) and the sealed mixture was shaken thoroughly and the solid lump broken with a glass rod. The alkaline solution was shaken with ether (50 ml.) and the total mixture filtered from dicyclohexylurea. The clear aqueous layer was washed twice with ether and kept for a total of 1 hr. at room temperature to remove the acetyl group. Amberlite 1R-120 (H⁺) resin was then added gradually until the pH dropped to neutrality and the solution was then filtered from resin, and the latter washed thoroughly with water. The total aqueous solution was concentrated at low temperature and made up to a standard volume and stored at 3°.

Polymerization of a Mixture of N,O-Diacetyldeoxycytidine-5' Phosphate and Thymidine-5' Phosphate.—The polymerization was carried out exactly as described above, except that one-half the scale was used. After working up by the addition of appropriate amount of aqueous alkali to the polymerization mixture and extraction with ether, the aqueous alkaline solution was passed through a column (6 cm. × 2 cm. dia.) of Amberlite-120 resin (ammonium form) and the total effluent and washings evaporated to dryness. The residue was dissolved in 10 ml. of conc. ammonia and the solution kept at room temperature for 2.5 hr. to ensure complete removal of the N-acetyl group. The solution was then evaporated and the residue made up to a standard volume in water and the solution stored at 3°.

Large-scale Separation of Polymers on DEAE-cellulose (Carbonate) Columns.—A portion of the solution (corresponding to 1 mmole of nucleotide) of the polymer mixtures obtained above was adjusted to pH 8-9 with ammonia and applied to the top of a DEAE-cellulose column (carbonate form) (30 cm. long × 4 cm. dia.) and carefully washed in with water (total volume of water wash, 300 ml.). Elution was begun using a linear gradient elution technique. In the case of polymers obtained from the mixture of 3'-O-acetylthymidine-5' phosphate and thymidine-5' phosphate, the mixing vessel contained initially 4 l. of water and the reservoir 4 l. of 0.25 M triethylammonium bicarbonate (pH 7.5). When this eluent had passed through the column, elution was continued by maintaining the linear gradient (4 l. of 0.25 M triethylammonium bicarbonate in the mixing vessel and 4 l. of 0.5 M salt in the reservoir). In the case of polymers from the mixture of N,O-diacetyldeoxycytidine-5'

phosphate and thymidine-5' phosphate, the same column was used but the linear gradient was a little different: first 4 l. of water in the mixing vessel and 4 l. of 0.3 M triethylammonium bicarbonate (pH 7.5) in the reservoir, and then 2 l. of 0.3 M triethylammonium bicarbonate in the mixing vessel and 2 l. of 0.45 M solution of the same salt in the reservoir. In both experiments, a flow rate of 2-2.3 ml./min. was maintained and approximately 20 ml. fractions were collected using an automatic fraction cutter. The elution pattern obtained from the polymers containing thymidine only is shown in Fig. 1, and the pooling of fractions and product composition is shown in Table I. The elution pattern obtained in the polymerization of a mixture of diacetyldeoxycytidine-5' phosphate and thymidine-5' phosphate is shown in Fig. 6 and the product distribution in Table V. The recovery of the nucleotide material in both experiments was essentially quantitative.

Each of the pooled peaks was evaporated *in vacuo* at low temperature and the residual syrup obtained, especially with higher oligonucleotides, was redissolved in water and the solution re-evaporated. The process was repeated several times to ensure complete removal of triethylammonium bicarbonate. Further processing of the peaks is described below. The concentrated solutions of those peaks that were to be purified by chromatography in ammoniacal solvents on paper strips were passed through small columns of ammonium Dowex-50 ion exchange resin to obtain ammonium salts of materials.

Further Purification of Thymidine Polynucleotides by Rechromatography on DEAE-cellulose (Carbonate) Columns.—The peaks from Fig. 1, corresponding to linear thymidine polynucleotides, were rechromatographed on DEAE-cellulose columns (20 cm. × 2 cm. diameter, average size) (carbonate form). Satisfactory results were obtained by pre-equilibrating the column with the triethylammonium carbonate concentration used in the mixing vessel and, by, applying the polynucleotide as a concentrated solution in the same salt solution. The elution was carried out by the linear gradient method using concentrations in the mixing vessel and reservoir as listed in Table II for each polynucleotide. On the average, 10-15 ml. fractions were collected, the flow rate being about 1 ml./min. The major peak was again recovered by evaporation of the combined eluate and repeated evaporation after addition of water. The triethylammonium salts of the polynucleotides were exchanged to ammonium salts by passage through small columns of ammonium Dowex-50 ion exchange resin and the solutions stored at 3°.

The number of minor peaks, obtained on rechromatography, in general, increased with the increasing size of the polynucleotide. The resolution was throughout satisfactory, as far as tried (undecanucleotide). The typical patterns obtained on rechromatography are illustrated with respect to the peak 12 (tetranucleotide) and peak 20 (decanucleotide) (Fig. 2 and 3 respectively). Rechromatography of the major peak of Fig. 3 gave a single sharp peak (Fig. 4).

Separation of Individual Polynucleotides Containing Terminal Deoxycytidine from Corresponding Polynucleotides Containing Thymidine Only.—Each of the major peaks from Fig. 6 corresponding to linear polynucleotides was rechromatographed on a DEAE-cellulose column (15 cm. × 2 cm. dia., average size) in the chloride form by using linear gradient elution technique. The volume used in the mixing vessel and in the reservoir was 1 l. each in all the experiments and the salt concentrations used are listed in Table VI. The flow rate was about 1.5 ml./min. and 15 ml. fractions were collected. Each of the original peaks gave two major peaks and often other minor peaks which were discarded. The first major peak was invariably the oligonucleotide containing terminal deoxycytidine, while the second major peak was the analogue containing thymidine only. The ratios of the optical density of the two major products obtained on rechromatography are also listed in Table VI. The typical elution patterns obtained are illustrated with respect to the dinucleotides (pTpC and pTpT) and heptanucleotides (pTpTpC and pTpTpT) in Fig. 7 and 8, respectively.

Recovery of Linear Polynucleotides.—The combined pure peak fractions obtained above from the chloride columns were neutralized with lithium hydroxide and then evaporated down to a completely solid white residue by first using a rotary evaporator and then sucking on an oil pump. The solid cake was dissolved in minimum of methyl alcohol

(2-4 ml.) and acetone (25-30 ml.) and then diethyl ether (5-10 ml.) added to precipitate the nucleotidic material. All the centrifuge tubes containing the precipitated materials were kept at 0° for some hours to ensure complete precipitation and the precipitates then centrifuged down. The precipitates were restirred by adding 1 ml. of methyl alcohol and then acetone (about 15 ml.) added. The precipitates were again collected by centrifugation. Finally they were washed with acetone and dried *in vacuo* at room temperature. The lithium salts of the polynucleotides thus obtained were dissolved in small amounts of water and converted to the ammonium salts by passing through columns (2 cm. X 1 cm. dia.) of ammonium Dowex-50 (200-400 mesh) ion exchange resin. The combined effluents and washings were concentrated to small volumes and the solutions stored frozen. Some loss was observed during recovery by the precipitation procedure with the lower thymidine oligonucleotides but in general the recoveries, especially with the polynucleotides containing terminal deoxycytidine were practically quantitative.

Purification of Cyclic Oligonucleotides.—Cyclic oligonucleotides (cyclic di- to penta-nucleotide) were purified for comparison with samples characterized earlier by prolonged paper chromatography in solvent A. The samples were applied by first exchanging the triethylammonium cation present in the concentrated solutions of the peaks (e.g., 4, 7, 10 and 13, of Fig. 1) for the ammonium ion by passing through ammonium Dowex-50 ion exchange resin columns.

Paper Chromatography.—Paper chromatography was carried out by the descending technique using double acid-washed paper (Whatman, paper 40 or 44). The solvent systems used are: solvent A, isopropyl alcohol-conc. ammonia-water (7-1-2, v./v.); solvent B, isobutyric acid-1 M ammonia-disodium ethylenediamine tetraacetate (0.1 M) (100-60-15, v./v.); solvent C, ethyl alcohol-1 M ammonium acetate (pH 7.5) (5-2, v./v.); solvent D, ethyl alcohol-0.5 M ammonium acetate (pH 3.8) (5-2, v./v.); solvent E, *n*-propyl alcohol-conc. ammonia-water (55-10-35, v./v.); solvent F, isopropyl alcohol-conc. ammonia-0.1 M boric acid (7-1-2, v./v.); solvent G, *n*-butyl alcohol-acetic acid-water (5-2-3, v./v.). The R_f 's of different polynucleotides in the solvents A-E which are the more useful are listed in Table III.

Paper Electrophoresis was carried out in an apparatus similar to that of Markham and Smith. The buffers used routinely were 0.05 M ammonium acetate (pH 3.2-3.5) and 0.05 M triethylammonium bicarbonate (pH 7.5). Thick double acid washed paper (Whatman #31) strips were used, the paper being soaked in the buffer and then blotted before application of the spots. The relative mobilities of different oligonucleotides are given in Table VII.

Enzyme Experiments. (a) **Removal of Terminal Phosphomonoester Groups.**—The prostatic phosphomonoesterase prepared by the method of Boman²⁸ was first used in early work under conditions of concentration, buffer and incubation as standardized with the dinucleotide pTpT being used. More recently the alkaline phosphatase of *Escherichia coli*, as prepared by Garen and Levinthal, has been used. The preparation containing about 2 mg./ml. of protein was diluted

fivefold with 0.05 M trihydroxymethyl aminomethane buffer (pH 8) and the conditions used for complete removal of phosphomonoester groups from mono- to deca-nucleotides were: substrate containing approximately 0.1 μ mole of phosphomonoester end group was contained in a final volume of 0.04-0.05 ml. of water. To it 0.002 ml. of 1 M trihydroxymethyl aminomethane buffer (pH 8) was added and the pH of the resulting solution checked to be around 8. 5 μ l. of the above diluted enzyme was added and the mixture incubated at 37° for 4 hr. Dephosphorylation of the phosphomonoester groups was complete in all the polynucleotides, and no contamination of any phosphodiesterase activity in the enzyme preparation was detected. The results obtained are illustrated with thymidine octa- to deca-nucleotides in Fig. 5, the chromatograms traced having been developed in solvent E for 5 1/2 days.

(b) **Degradation of Polynucleotides by Venom Phosphodiesterase.**—Aliquots of solutions of the purified polynucleotides (homologous series of general structure III) containing a total of about 0.5 μ mole of the nucleoside were lyophilized in small reaction tubes. To the residue in each tube was added 0.01 ml. of 2 M ammonium carbonate, pH 9, buffer and 0.04 ml. of venom phosphodiesterase preparation. The enzyme preparation was combined peak tubes from DEAE-cellulose column chromatography after acetone fractionation, as described previously.²⁹ The incubations were carried out at 37° for 6-7 hr. in all cases. Under these conditions, degradation was complete in every case. (Previously the venom diesterase preparation had been standardized with respect to pTpT and TpT; the incubation time given in the degradation of polynucleotides and the amount of enzyme used were each double that necessary for the complete hydrolysis of the reference substrates.) The total incubation mixtures were then applied on paper chromatograms which were developed in solvent C. The spots and appropriate blanks were eluted by soaking in 0.1 N hydrochloric acid for at least 18 hr. The ratios of deoxycytidine-5' phosphate to thymidine-5' phosphate are listed in Table VIII.

Exactly the same procedure was used for degradation of thymidine polynucleotides of general structure IV (pTpT to thymidine-5' phosphate and thymidine, the total incubation mixtures being applied on paper chromatograms which were developed in solvent A). The ratios of the nucleotide to the nucleoside are listed in Table IV by first identifying the N-Pyridinium Nucleotide (Peak 1, Fig. 1). The major constituent of this peak showed the following characteristics: Its mobility on paper electrophoresis at pH 3.5 was nil. At pH 7.5 it had a net negative charge, electrophoretic mobility being 0.46 that of thymidine-5' phosphate. On paper chromatograms in solvent A, its R_f was 0.55 relative to thymidine-5' phosphate. It was dephosphorylated by prostatic phosphomonoesterase and the resulting ultraviolet absorbing material had R_f in solvent A of 0.38 (R_f of thymidine run as marker, 0.65). The substance moved toward the cathode on paper electrophoresis, showing positive charge. The ultraviolet absorption spectrum of the substance showed a peak at 260 m μ with a shoulder (almost a second peak) at 267 m μ .

(27) R. Markham and J. D. Smith, *Biochem. J.*, **52**, 552 (1952).

(28) H. G. Boman, *Arkiv. Kemi.*, **12**, 453 (1958).

(29) W. E. Razzell and H. G. Khorana, *J. Biol. Chem.*, **234**, 2105 (1959).

- ⁵ Plescia, O. J., J. Noval, N. C. Palczuk, and W. Braun, *Proc. Soc. Exptl. Biol. Med.*, 106, 748 (1961).
- ⁶ Palczuk, N. C., O. J. Plescia, and W. Braun, *Proc. Soc. Exptl. Biol. Med.*, 107, 982 (1961).
- ⁷ Deicher, H. R. G., H. R. Holman, and H. G. Kunkel, *J. Exptl. Med.*, 109, 97 (1959).
- ⁸ Stollar, D., L. Levine, H. I. Lehrer, and H. Van Vunakis, these PROCEEDINGS, 48, 874 (1962).
- ⁹ Levine, L., W. T. Murakami, H. Van Vunakis, and L. Grossman, these PROCEEDINGS, 46, 1038 (1960).
- ¹⁰ Townsend, E., W. T. Murakami, and H. Van Vunakis, *Federation Proc.*, 20, 438 (1961).
- ¹¹ Butler, V. P., Jr., S. Beiser, B. F. Erlanger, S. W. Tanenbaum, S. Cohen, and A. Bendich, these PROCEEDINGS, 48, 1597 (1962).
- ¹² Tanenbaum, S. W., and S. M. Beiser, these PROCEEDINGS, 49, 662 (1963).
- ¹³ Goebel, W. F., and O. T. Avery, *J. Exptl. Med.*, 54, 437 (1931).
- ¹⁴ Sueoka, N., and T. Cheng, *J. Mol. Biol.*, 4, 161 (1962).
- ¹⁵ Plescia, O. J., K. Amiraian, and M. Heidelberger, *J. Immunol.*, 78, 147 (1957).
- ¹⁶ Doty, P., J. Marmur, J. Eigner, and C. Schildkraut, these PROCEEDINGS, 46, 461 (1960).
- ¹⁷ Heidelberger, M., and F. E. Kendall, *J. Exptl. Med.*, 54, 515 (1931).
- ¹⁸ Meselson, M., F. W. Stahl, and J. Vinograd, these PROCEEDINGS, 43, 581 (1957).

URIDINE-SPECIFIC ANTIBODIES OBTAINED WITH SYNTHETIC ANTIGENS*

BY MICHAEL SELA, HANNA UNGAR-WARON, AND Yael SHECHTER

SECTION OF CHEMICAL IMMUNOLOGY, THE WEIZMANN INSTITUTE OF SCIENCE, REHOVOTH, ISRAEL

Communicated by Christian B. Anfinsen, June 10, 1964

The problem of the antigenicity of nucleic acids has been approached from several directions with varying degrees of success.¹ Reports in the older literature² on antibodies with specificity directed toward nucleic acids have been challenged because of doubts concerning the purity of the nucleic acid used either for immunization or for specific reaction with the antibodies formed. Neither RNA¹ nor DNA^{3, 4} nor synthetic polynucleotide⁵ preparations were found to be immunogenic by the serological methods employed. On the other hand, positive results were reported with DNase-sensitive antigens from *Brucellae*^{6, 7} and with a soluble RNA preparation from yeast.⁸ Antibodies directed toward thermally denatured DNA have been detected in rabbit antisera to ruptured T-even coliphage⁹ and in sera of patients with lupus erythematosus.¹⁰⁻¹² In the case of the coliphage the antibodies were shown to be directed, in part, toward the glucosylated 5-hydroxymethylcytosine.¹³ Antibodies with specificity toward RNA were also detected in antisera to bacterial ribosomes.¹⁴⁻¹⁶

An alternative approach to the elucidation of immunological properties of nucleic acids consists of efforts to bind, chemically, their components to well-defined antigens, and to study the specificity of antibodies elicited by means of such artificial conjugates. Thus, antibodies with purine or pyrimidine specificities, reacting with heat-denatured DNA, were obtained in rabbits upon injection of purinoyl¹⁷ or uracil-conjugates¹⁸ of serum albumins.

This report describes the chemical binding of a uridine derivative to two different multichain synthetic polypeptides, one antigenic and the other nonantigenic.^{19, 20} The injection into rabbits of these synthetic nucleoside-polypeptide conjugates

elicited, in both cases, antibodies with specificity toward uridine, and which reacted with single-stranded thymus DNA, heat-denatured *E. coli* RNA, and polyribouridylic acid.

Materials and Methods.—Nucleosides and polyadenylic acid were obtained from Sigma Chemical Company, uracil and d-ribose from Nutritional Biochemicals, and calf thymus DNA from Worthington Biochemical Corp. We are indebted to Dr. S. Ochoa for a gift of polyuridylic acid, and to Dr. U. Z. Littauer for a gift of *E. coli* RNA.

The multichain polymer multi-poly-DL-alanyl—poly-L-lysine (pAla—pLys) was prepared¹⁸ from N-carboxy-DL-alanine anhydride and poly-L-lysine, in a residue molar ratio of Ala:Lys, 7:1. Uridine-5'-carboxylic acid and thymidine-5'-carboxylic acid were synthesized according to Moss *et al.*²¹ N,N'-dicyclohexylcarbodiimide (0.5 gm, Fluka, Switzerland) was added to a mixture of pAla—pLys (1.5 gm in water) and uridine-5'-carboxylic acid (0.75 gm in dimethylformamide). The water content of the final reaction mixture was 5%. After 18 hr, it was dialyzed against distilled water (3 days), filtered, and freeze-dried. The resulting U-pAla—pLys (Fig. 1) contained 9% uridine-5'-CO-, determined from the extinction at 260 m μ (corresponding to a molar ratio of U:Ala:Lys, 1:32:4.5), and had a molecular weight of 80,000, calculated from a sedimentation coefficient of $s_{20,w} = 4.3$ S (1% solution in 0.9% sodium chloride), a diffusion coefficient of $D_{20,w} = 4.7 \times 10^{-7}$ cm² sec⁻¹, and a partial specific volume of 0.72. A thymidine derivative, T-pAla—pLys, was prepared analogously (8.5% thymidine-5'-CO-).

In order to obtain a multichain polymer that would contain both uridine and tyrosine, use was made of the observation that U-pAla—pLys still contained unreacted amino groups. It was, therefore, reacted (0.8 gm in 0.05 M phosphate buffer, pH 7.0) with N-carboxy-L-tyrosine anhydride (0.2 gm in dioxane); after 24 hr at 2°, the product was dialyzed against distilled water (3 days) and freeze-dried. The resulting (U,pTyr)-pAla—pLys contained 14.5% tyrosine residues (from the extinction at 293.5 m μ and pH 13, as well as ninhydrin colorimetry after hydrolysis and paper chromatography¹⁹). The molar ratio of U:Tyr:Ala:Lys was 1:3.2:32:4.5. The polymer had a sedimentation coefficient $s_{20,w} = 4.7$ S.

Calf thymus DNA and *E. coli* RNA were denatured by boiling aqueous solutions for 10 min followed by rapid cooling in an ice bath. DNA was also heat-denatured in 1% formaldehyde.²²

Immunological procedures: The polymers tested did not give any precipitate with preimmunization sera. Groups of eight rabbits were immunized (using Freund's adjuvant) as described previously.¹⁹ All experiments were carried out with pooled antisera. Precipitin and inhibition tests were performed as described by Fuchs and Sela.²⁰

Results.—The homologous precipitin reaction of the system U-pAla—pLys-

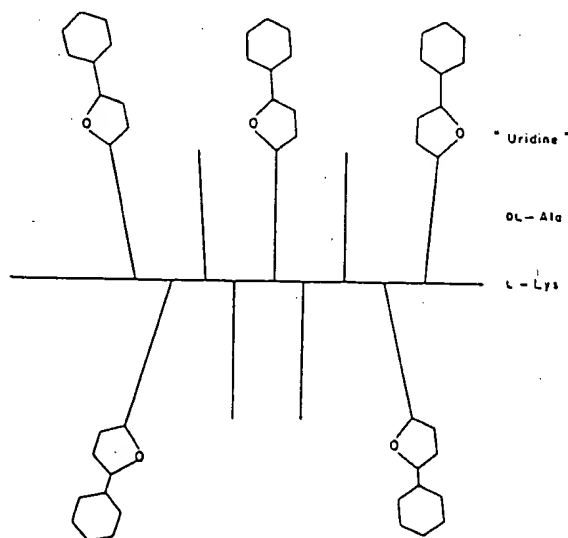


FIG. 1.—Schematic presentation of the multichain nucleoside-polypeptide conjugate U-pAla—pLys.

anti-U-pAla—pLys is shown in Figure 2. The antisera cross-precipitated partially with T-pAla—pLys, and slightly with pAla—pLys. In the last case, the maximal precipitation occurred at a much higher concentration of the polymer. The homologous reaction was also positive when checked by the passive cutaneous anaphylaxis technique.²³

The specificity of the antibodies obtained is apparent from inhibition studies. Neither uracil (up to 5 mg/ml serum) nor d-ribose (up to 10 mg/ml) nor a mixture of the two had any inhibitory effect on the homologous precipitin reaction. On the other hand, almost total inhibition was observed with uridine, and a partial one with thymidine (Fig. 3). Cytidine, at 5 mg/ml serum, caused 20 per cent inhibition. No inhibition was found with guanosine (0.5 mg/ml) or adenosine (1 mg/ml). The last two nucleosides are not well soluble at higher concentrations. Uridine did not affect the extent of precipitation of egg albumin with anti-egg albumin. Nucleoside-5'-carboxylic acids were not used in inhibition studies, as uridine-5'-carboxylic acid inhibited efficiently the homologous systems of both U-pAla—pLys and egg albumin. On the other hand, uridine-3'-phosphoric acid caused 50 per cent inhibition at 1 mg/ml antiserum, but did not inhibit at all the homologous egg albumin-anti-egg albumin system.

The cross-reaction of the antiuridine antibodies with nucleic acids was also investigated. Native calf thymus DNA did not cross-precipitate with the antiserum, while heat-denatured DNA gave a typical precipitin reaction, and an even better cross-precipitation was obtained with DNA heat-denatured in the presence of formaldehyde (Fig. 4).

Neither native nor heat-denatured *E. coli* RNA cross-precipitated with the anti-

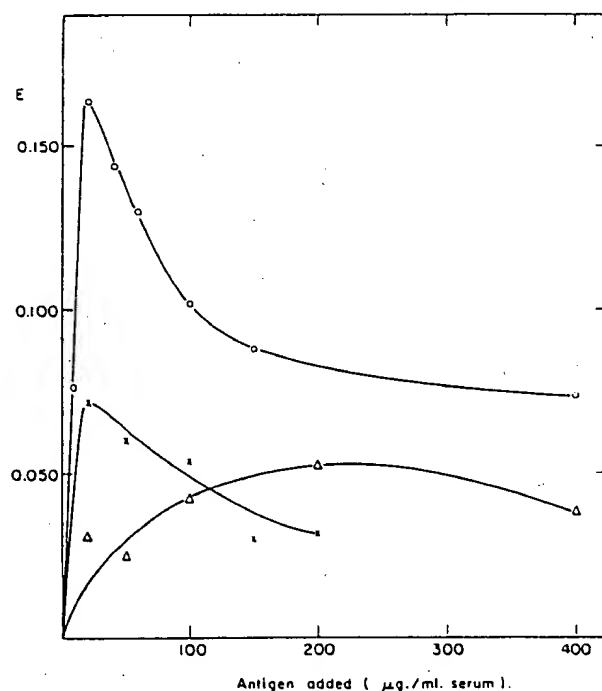


FIG. 2.—Extinction at 2800 Å of solutions in 0.1 *N* sodium hydroxide of precipitates obtained by the addition to antiserum against U-pAla—pLys of: O, U-pAla—pLys; X, T-pAla—pLys; Δ, pAla—pLys.

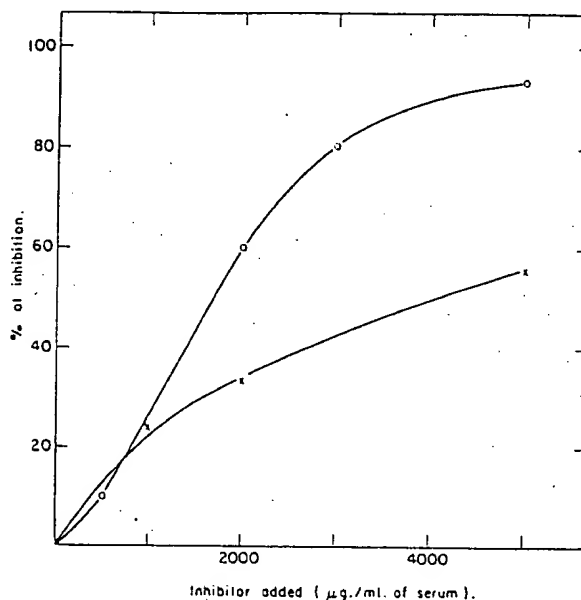


FIG. 3.—Inhibition curves of the homologous reaction of the system U-pAla—pLys and its anti-serum by: O, uridine; X, thymidine (antigen conc. 20 μ g/ml serum).

uridine antiserum. This was due to the presence of RNase in the serum (rabbit serum contains RNase equivalent to approximately 0.1 μ g bovine pancreatic RNase per ml²⁴). The RNase action was inhibited by the addition of 200 μ g of γ -globulin isolated on DEAE-cellulose²⁵ from an anti-RNase antiserum per 1 ml of antiuridine serum. When after 24 hr RNA was added, cross-precipitation was obtained with the heat-denatured material, but not with the native *E. coli* RNA (Fig. 5). Polyuridylic acid gave a precipitate with the antiuridine serum in the presence of anti-RNase γ -globulin, in contrast to polyadenylic acid (Fig. 5). None of the nucleic acid samples mentioned above gave, at the concentrations used, any precipitation with either normal rabbit sera or antiegg albumin sera.

In Figures 6 and 7 are shown, respectively, some precipitin and inhibition reactions of the antiserum to (U,pTyr)-pAla—pLys. In this case, beside specific antiuridine antibodies, antipolypeptide antibodies were also formed, as apparent from the incomplete inhibition of the homologous reaction by uridine (Fig. 7).

Discussion.—The experiments described show that antibodies with specificity

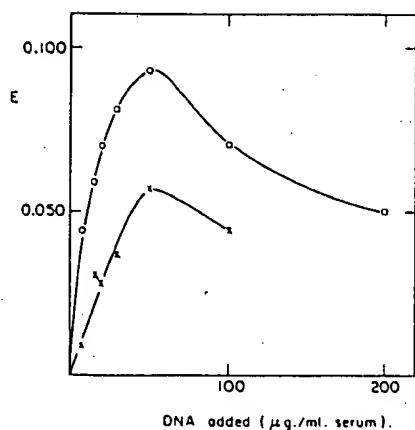


FIG. 4.—Extinction at 2800 Å of solutions in 0.1 N sodium hydroxide of precipitates obtained by the addition to antiserum against U-pAla—pLys of: X, heat-denatured DNA; O, DNA heat-denatured in the presence of 1% formaldehyde.

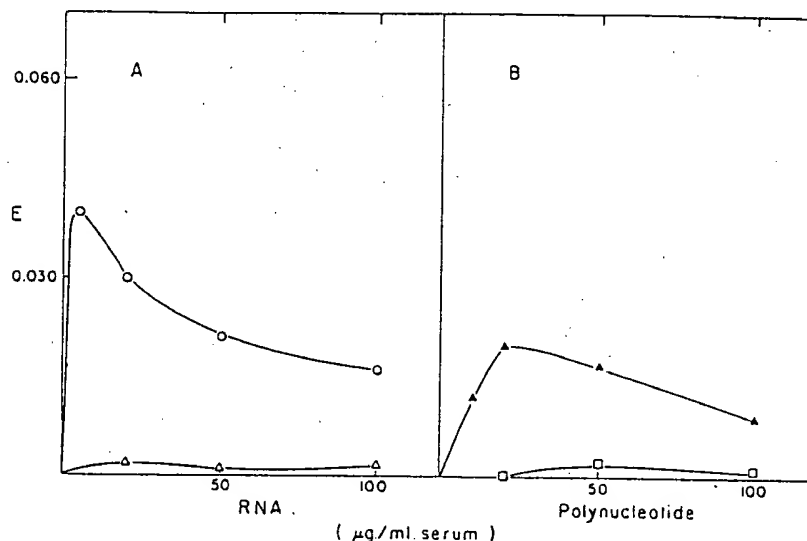


FIG. 5.—Extinction at 2800 Å of solutions in 0.1 *N* sodium hydroxide of precipitates obtained by the addition to antiserum against U-pAla—pLys previously treated with 200 μg/ml serum anti-RNAase γ-globulins of: (A) O, heat-denatured RNA; Δ, native RNA. (B) ▲, polyuridylic acid; □, polyadenylic acid.

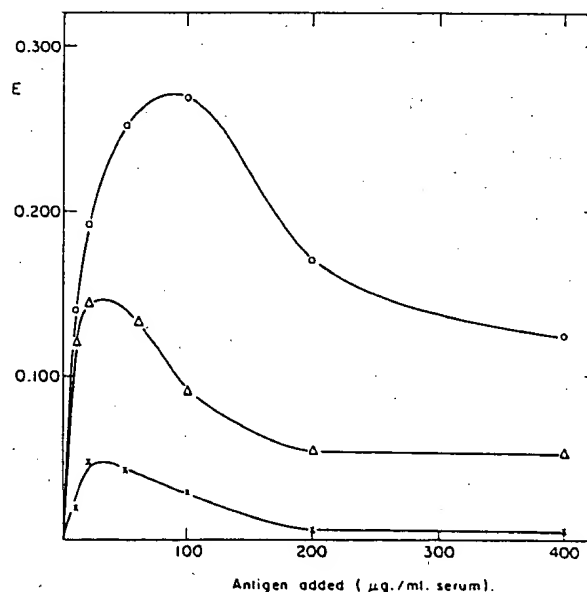


FIG. 6.—Extinction at 2800 Å of solutions in 0.1 *N* sodium hydroxide of precipitates obtained by the addition to antiserum against (U, pTyr)-pAla—pLys of: O, (U, pTyr)-pAla—pLys; Δ, U-pAla—pLys; X, pAla—pLys.

toward uridine may be obtained in rabbits upon injection of synthetic molecules in which uridine-5'-carboxylic acid is bound through an amide bond to the amino-terminal groups of poly-DL-alanyl side-chains of a multichain synthetic polypeptide. The attachment of the uridine-5'-carboxylic acid residues not only changed extensively the specificity of an antigenic synthetic polypeptide (Fig. 6), but also converted the nonantigenic¹⁹ multichain poly-DL-alanine into an immunogen (Fig. 2), with specificity due mostly to uridine. While some anti-poly-DL-alanine was

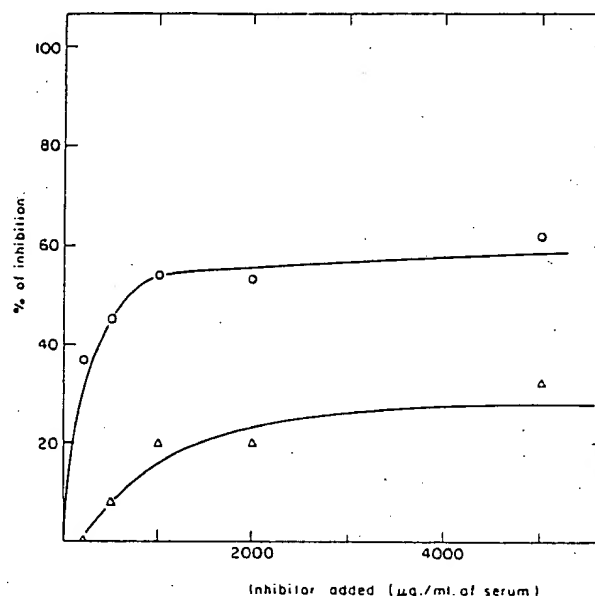


FIG. 7.—Inhibition curves of the homologous reaction of the system (U,pTyr)-pAla—pLys and its antiserum by: O, uridine; Δ, thymidine (antigen conc. 40 μg/ml serum).

formed, the antibodies in the equivalence zone (ca. 120 μg/ml serum) consisted almost entirely of antiuridine, as apparent from inhibition studies (Fig. 3).

The antigenic specificity of U-pAla—pLys is due to the nucleoside unit as a whole, since uracil and/or ribose did not inhibit the homologous reaction, while uridine and uridylic acid were efficient inhibitors. The inhibition with thymidine demonstrates that neither the methyl group in position 5 of the pyrimidine ring nor the hydroxyl in position 2 of the ribose are of paramount importance in defining the combining sites of the antibodies formed. The lack of inhibition by the purine nucleosides stresses the role of the pyrimidine ring in the specificity. Nucleoside-5'-carboxylic acids inhibited efficiently both the uridine-specific homologous system and the egg albumin-anti-egg albumin system. This is in agreement with a previous report²⁶ that heterocyclic carboxylic acids inhibit precipitin reactions.

The specificity toward uridine of the antibodies obtained is also apparent from the cross-precipitation with polyuridylic acid, but not with polyadenylic acid. In order to obtain precipitin reactions, RNase activity of the sera²⁴ had to be neutralized with antibodies against the enzyme. By this technique the reaction of heat-natured *E. coli* RNA, but not of native RNA, with antibodies toward uridine could also be demonstrated (Fig. 5). The lack of interaction of antibodies to purinoyl and uracil conjugates of proteins with RNA^{17, 18} may thus have been due to the presence of RNase in the sera tested. In studies of antiribosomal antibodies, Barbu and Dandeu²⁷ have used bentonite to remove RNase from the sera. The cross-precipitations, reported here, with RNA and polyuridylic acid may be incomplete, as it is possible that not all RNase activity was removed from the sera tested.

Even though DNA does not contain uridine, a single-stranded calf thymus DNA preparation (but not the double-stranded DNA) cross-precipitated with the test antiserum, probably because of its thymidine content. This observation is similar to the report of cross-reaction of antibodies against a uracil-protein conjugate with

single-stranded DNA.¹⁸ The lack of reactivity of double-stranded DNA with the antiuridine sera is in agreement with previous reports^{9, 11, 17, 18} of the preferential reactivity of antisera specific toward nucleic acid or their components, with single-stranded rather than double-stranded DNA. Apparently, the antigenic determinants are not available in the highly ordered structures of double-stranded DNA or the high molecular weight *E. coli* RNA,²⁸ for the reaction with nucleoside-specific antibodies. While the heat-denaturation of *E. coli* RNA is a reversible phenomenon,²⁸ the cross-reaction with the antiuridine serum suggests that the renaturation upon quick cooling is not complete. On the other hand, it was reported recently that polyuridylic acid, which cross-reacted with antiuridine, is a randomly coiled polynucleotide.²⁹

The induction with fully synthetic antigens of biosynthesis of antibodies specific toward uridine permits a systematic investigation of the role of various molecular parameters in the immunogenicity and antigenic specificity of nucleoside-containing synthetic macromolecules. Preliminary results indicate that antibodies with specificity toward thymidine were obtained upon injection into rabbits of T-pAla—pLys, and the synthesis and immunochemical characterization of other potential nucleoside-specific immunogens is in progress. The availability of antibodies with specificity toward nucleosides and nucleotides should be helpful in investigations of the manifold chemical, physical, and biological properties of nucleic acids.

Summary.—Completely synthetic antigens obtained by the chemical binding of uridine-5'-carboxylic acid to synthetic multichain polypeptides have elicited, in rabbits, antibodies with specificity toward uridine, as apparent from cross-precipitation and inhibition reactions. The attachment of the uridine derivative to a nonantigenic macromolecule converted it into an immunogen. The antibodies formed reacted with polyuridylic acid, heat-denatured RNA and DNA, but not with polyadenylic acid, native *E. coli* RNA, or double-stranded calf thymus DNA.

* This investigation was supported in part by the USPHS research grant AI-04715 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

¹ Pasternak, G., F. Hoffman, H. Bielka, and L. Venker, *Pathol. Microbiol.*, **23**, 392 (1960).

² Lackman, D., S. Mudd, M. G. Sevag, J. Smolens, and M. Wiener, *J. Immunol.*, **40**, 1 (1941).

³ Colter, J. S., *Nature*, **190**, 550 (1961).

⁴ Yachnin, S., *J. Immunol.*, **91**, 529 (1963).

⁵ Yachnin, S., *Nature*, **195**, 1319 (1962).

⁶ Phillips, J. H., W. Braun, and O. J. Plescia, *Nature*, **181**, 573 (1958).

⁷ Olitzki, A. L., *Brit. J. Exptl. Pathol.*, **41**, 623 (1960).

⁸ Bigley, N. J., M. C. Dodd, and V. B. Geyer, *J. Immunol.*, **90**, 416 (1963).

⁹ Levine, L., W. T. Murakami, H. Van Vunakis, and L. Grossman, these PROCEEDINGS, **46**, 1038 (1960).

¹⁰ Deicker, H. R. G., H. R. Holman, and H. G. Kunkel, *J. Exptl. Med.*, **109**, 97 (1959).

¹¹ Stollar, D., and L. Levine, *J. Immunol.*, **87**, 477 (1961).

¹² Stollar, D., L. Levine, H. J. Lehrer, and H. Van Vunakis, these PROCEEDINGS, **48**, 874 (1962).

¹³ Murakami, W. T., H. Van Vunakis, H. J. Lehrer, and L. Levine, *J. Immunol.*, **89**, 116 (1962).

¹⁴ Barbu, E., J. Panijel, and G. Quash, *Ann. Inst. Pasteur*, **100**, 725 (1961).

¹⁵ Barbu, E., G. Quash, and J.-P. Dandeu, *Ann. Inst. Pasteur*, **105**, 849 (1963).

¹⁶ Panijel, J., *Compt. Rend. Acad. Sci.*, **256**, 4540 (1963).

¹⁷ Butler, V. P., S. M. Beiser, B. F. Erlanger, S. W. Tanenbaum, S. Cohen, and A. Bendich, these PROCEEDINGS, **48**, 1597 (1962).

¹⁸ Tanenbaum, S. W., and S. M. Beiser, these PROCEEDINGS, **49**, 662 (1963).

- ¹⁹ Sela, M., S. Fuchs, and R. Arnon, *Biochem. J.*, **85**, 223 (1962).
- ²⁰ Fuchs, S., and M. Sela, *Biochem. J.*, **87**, 70 (1963).
- ²¹ Moss, G. P., C. B. Reese, K. Schofield, R. Shapiro, and Lord Todd, *J. Chem. Soc.*, 1149 (1963).
- ²² Stollar, D., and L. Grossman, *J. Mol. Biol.*, **4**, 31 (1962).
- ²³ Ovary, Z., *Progr. Allergy*, **5**, 459 (1958).
- ²⁴ Philipson, L., and M. Kaufman, *Biochim. Biophys. Acta*, **80**, 151 (1964).
- ²⁵ Levy, H. B., and H. A. Sober, *Proc. Soc. Exptl. Biol. Med.*, **103**, 250 (1960).
- ²⁶ Kleinschmidt, W. J., and P. D. Boyer, *J. Immunol.*, **69**, 247 (1952).
- ²⁷ Barbu, E., and J.-P. Dandeu, *Compt. Rend. Acad. Sci.*, **256**, 1166 (1963).
- ²⁸ Cox, R. A., and U. Z. Littauer, *Biochim. Biophys. Acta*, **61**, 197 (1962).
- ²⁹ Richards, E. G., P. Flessel, and J. R. Fresco, *Biopolymers*, **1**, 431 (1963).

THE ENZYMATIC METHYLATION OF RNA AND DNA, VIII.
EFFECTS OF BACTERIOPHAGE INFECTION ON THE ACTIVITY OF
THE METHYLATING ENZYMES*

BY MARVIN GOLD, RUDOLF HAUSMANN,† UMADAS MAITRA,‡ AND JERARD HURWITZ§

DEPARTMENT OF MOLECULAR BIOLOGY, ALBERT EINSTEIN COLLEGE OF MEDICINE

Communicated by B. L. Horecker, June 11, 1964

We, as well as others, have previously reported on the presence in *Escherichia coli* of several enzymes which catalyze the transfer of methyl groups from S-adenosylmethionine to sRNA,²⁻⁴ ribosomal RNA,^{5, 6} and DNA.^{4, 7} Although the biological function of the methylated bases which these enzymes produce is still obscure, the species and strain specificity of the methylation reactions suggest that they provide a basis for a recognition mechanism. The virulent bacteriophage-host cell system is an example of a phenomenon involving recognition by the host of a foreign nucleic acid; in some instances, phage DNA is rapidly synthesized while the host DNA is rapidly degraded. If methylated bases are involved in controlling such a recognition mechanism, then a study of the methylated base content of DNA's of various bacteriophages grown in different hosts might provide a clue as to the biological function of the methylating enzymes. In order to establish a suitable system for further investigation, we have studied the effects of phage infection on the activities of the various methylating enzymes in the host cell. This communication summarizes such studies. It has been found that while the RNA methylases are apparently unchanged, DNA methylation activity increases markedly after infection with T2. In contrast, T3 infection induces an enzyme which cleaves S-adenosylmethionine to thiomethyladenosine and homoserine.

Materials and Methods.—(a) *Bacteria and phage:* *E. coli* B, used for infection experiments with the T series of bacteriophage was a strain obtained from Dr. C. Bresch of the University of Cologne. *E. coli* K12 strain W3104 and its lysogenic variant, W3104 (λ) were obtained from Dr. A. D. Kaiser of Stanford University and were used for studies on the effects of infection with or induction of bacteriophage λ, respectively.

Phages T1, T2, T4, T5, and T6 were generously provided from the stocks of the Department of Microbiology, New York University School of Medicine. Phages T3 and T7 were gifts of Drs. R. Latarjet of the Pasteur Institute and C. Bresch, respectively. Bacteriophage λ stocks were prepared from a single plaque isolated after plating the supernatant medium of an *E. coli* K12 (λ) culture on *E. coli* K12.

ANTIBODIES TO TRANSFER RNA OBTAINED WITH COVALENTLY LINKED tRNA CONJUGATES

Benjamin Bonavida, Sara Fuchs and Michael Sela

Department of Chemical Immunology
The Weizmann Institute of Science, Rehovot, Israel

Received November 3, 1970

SUMMARY

Precipitating antibodies with specificity directed towards transfer RNA were obtained in rabbits and goat upon immunization with conjugates of transfer RNA bound covalently either to bovine serum albumin or to a synthetic antigen by means of a water-soluble carbodiimide. Radioactive transfer RNA was retained specifically on a column of anti-tRNA-Sepharose and displaced with unlabeled tRNA.

INTRODUCTION

Antibodies which react with nucleic acids have been produced experimentally by immunization with ruptured bacteriophage, ribosomes, bases and derivatives chemically coupled to proteins or multichain polypeptides as well as with electrostatic complexes between methylated bovine serum albumin (MBSA) and DNA or synthetic polynucleotides (1,2). The production of antibodies to transfer RNA following the immunization of one rabbit with yeast tRNA in complete Freund's adjuvant was reported by Bigley *et al.* (3). However, Hernandez *et al.* demonstrated that antibodies produced in rabbits immunized with yeast tRNA were directed only to the contaminant oligonucleotides present in the tRNA preparation (4). Plescia *et al.* immunized rabbits with a complex of tRNA and MBSA (5). The antibodies obtained reacted with tRNA from various species, as followed by complement fixation.

In order to elucidate the role of tRNA conformation in its antigenic specificity, we were interested in obtaining precipitating anti-tRNA antibodies. The present report describes the preparation of conjugates of tRNA covalently bound to a protein or to a polypeptide by using a water-soluble carbodiimide reagent. Rabbits and a goat were immunized with such conjugates. For comparison, rabbits were also immunized with a complex of tRNA and MBSA as well as with tRNA alone. Specific precipitating antibodies to tRNA were obtained only upon immunization with covalently bound tRNA.

MATERIALS AND METHODS

Yeast tRNA (Boehringer, Mannheim) was extensively dialyzed and chromatographed on a Sephadex G-25 column to free the preparation of oligonucleotide contaminants. *E. coli* tRNA and *E. coli*-³²P-tRNA were a gift from Dr. V. Daniel.

Preparation of tRNA conjugates — Bovine serum albumin (BSA) (crystallized Armour) in 0.1 M sodium acetate, pH 5.5, was treated with 0.15 M iodoacetic acid to inactivate any residual ribonuclease activity (6). The preparation was dialyzed against water and lyophilized. Twenty mg BSA were dissolved in 2 ml of 0.05 M Tris-HCl, pH 7.5. To this solution, yeast tRNA (50 mg) in 2 ml of Tris-HCl buffer, pH 7.5 was added, and the mixture was adjusted to pH 7.5 with 0.1 N NaOH. Forty mg of 1-ethyl (3-dimethylaminopropyl)-carbodiimide HCl (EDCI) (Ott, Muskegon) in 1.0 ml water was added, and the reaction mixture was incubated at room temperature for 18 hours. The solution was then chromatographed on a Sephadex G-200 column (75x2 cm) equilibrated with 0.01 M Tris-HCl buffer, pH 7.5. The peak eluted at the void volume was collected and lyophilized. Protein was determined according to Lowry *et al.* (7), and tRNA from optical density at 260 and 280 nm. tRNA was also conjugated to the synthetic antigen poly(Glu, Tyr)-polyDLAla--polyLys (abbreviated as (T, G)-A--L), following the same procedure as described above for the preparation of the tRNA-BSA conjugate. The preparation of (T, G)-A--L used in this study had an average molecular weight of 180 and a residue molar ratio of $\underline{\text{L}}\text{-Lys}:\underline{\text{L}}\text{-Tyr}:\underline{\text{L}}\text{-Glu}:\underline{\text{DL}}\text{-Ala}$, 1:0.9:1.8:17.2. A conjugate of tRNA with MBSA was prepared according to Plescia *et al.* (5).

Immunological and chemical procedures — Several rabbits and a goat were immunized each with 2 mg antigen emulsified in complete Freund's adjuvant intradermally in multiple sites. The animals were injected 10 days later and bled at weekly intervals following the second immunization. Immune precipitation and inhibition of precipitation were done as described previously (8). tRNA, the conjugate tRNA-(T, G)-A--L and gamma globulin (prepared by precipitation with ammonium sulfate at 40% saturation) were coupled to Sepharose (9, 10) as follows: 5.0 g (net weight) of washed Sepharose 4B (Pharmacia, Uppsala) were suspended in 15 ml water and 0.5 g CNBr (Eastman Kodak) was added. The pH of the suspension was adjusted to 11.0, and was kept at this value for 8-10 minutes by the addition of 2 N NaOH. The reaction was terminated by filtration and washing several times with cold water. The activated Sepharose was washed with 0.1 M NaHCO₃. Thirty mg of tRNA, tRNA-(T, G)-A--L or rabbit gamma globulin were dissolved in 5 ml of 0.1 M NaHCO₃, and to each solution 1 g of the activated Sepharose was added. After 16 hours of gentle stirring at 4°C,

suspensions were filtered and washed several times with 0.1 M NaHCO_3 until the absorbance at 280 nm or 260 nm of the washing fluids was less than 0.02. The conjugated Sepharose was then equilibrated with 0.14 M NaCl -0.01 M phosphate buffer, pH 7.4 (PBS). From the absorbance of the material before and after the coupling to Sepharose, it was estimated that 90% of the gamma globulin, and 50% of both tRNA and tRNA-(T, G)-A--L were covalently bound to the Sepharose. The Sepharose conjugates were packed in Pasteur pipettes and were used as immunoadsorbent columns.

RESULTS AND DISCUSSION

Figure 1 illustrates a representative gel filtration on Sephadex G-200, for the separation of tRNA-BSA conjugate from the unreacted materials. Two peaks were obtained. The first peak, emerging in the void volume, was not observed in control experiments when preparations of either BSA or tRNA were treated with EDCI alone. Both BSA and tRNA elute in the region of the second peak. The presence of tRNA and protein in the high molecular weight material in the first peak was apparent both from the high absorbance at 260 nm and by the positive Lowry reaction. When this material was treated with bovine pancreatic ribonuclease and rechromatographed on a Sephadex G-200 column the first peak disappeared, and only the second peak was obtained. Similar chromatograms were obtained with the tRNA-(T, G)-A--L.

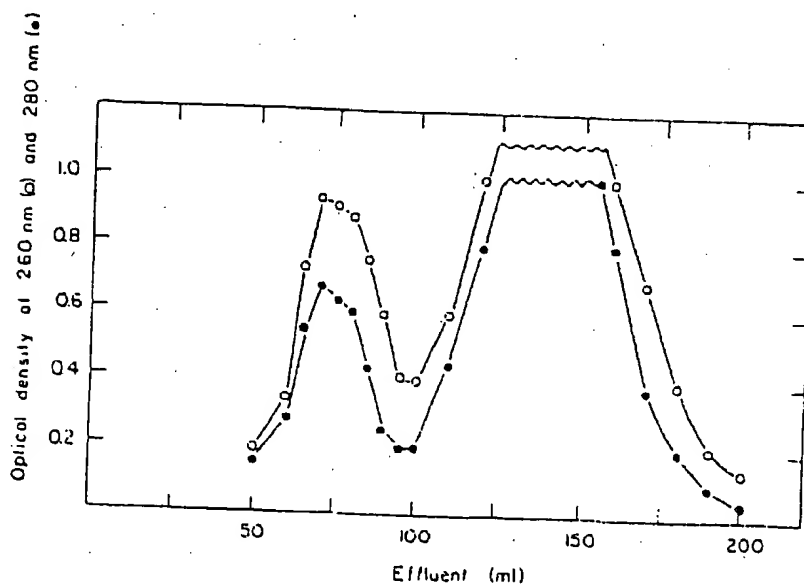


Fig. 1: Separation of tRNA-BSA conjugate from the unreacted materials on a Sephadex G-200 column (75x2 cm). Elution with 0.01 M Tris-HCl buffer, pH 7.6

Coupling of tRNA to a protein probably takes place via the terminal 5'-phosphate group of tRNA and the free amino groups of the protein. Halloran and Parker (11) used EDCI for the coupling of mononucleotides to BSA, and demonstrated that the amino group of the protein reacts with the 5'-phosphate group of the nucleotide to form a P-N bond. Both adenosine and adenine reacted poorly under the usual conditions of coupling.

Antibodies to tRNA — Groups of 4 rabbits were immunized, respectively, with tRNA-BSA, tRNA alone and the complex of tRNA and MBSA. Antibodies to tRNA were observed in the sera of all four animals immunized with the tRNA-BSA conjugate. Antibodies (0.2 mg/ml serum) precipitated with the heterologous tRNA-(T, G)-A--L conjugate. Likewise, a goat that was immunized with tRNA-(T, G)-A--L elicited antibodies that precipitated with the tRNA-BSA conjugate. Figure 2 illustrates a representative precipitin curve of an antiserum against tRNA-BSA with tRNA-(T, G)-A--L. The precipitate could be completely inhibited with tRNA.

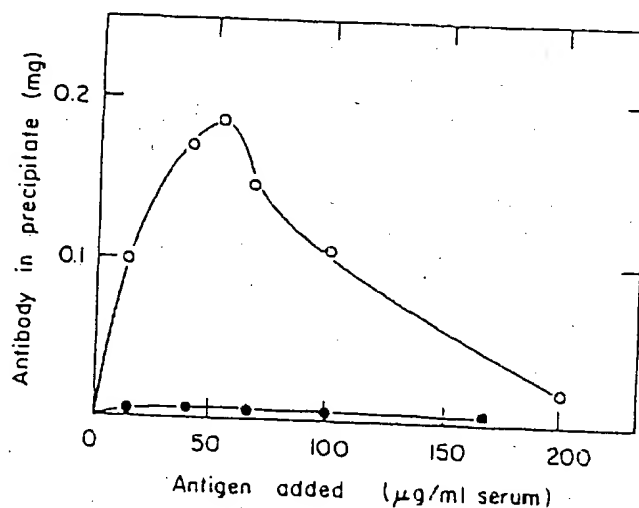


Fig. 2: Precipitin reaction with tRNA-(T, G)-A--L. tRNA-(T, G)-A--L was added to: O, rabbit anti-tRNA-BSA; ●, rabbit normal serum. The amount of the antibodies in the immune precipitate was determined from the absorbance at 280 nm of the dissolved precipitates after subtracting the contribution of the antigen.

Table 1 summarizes the precipitating capacity of the various antisera prepared against the tRNA-BSA conjugate, the tRNA-MBSA complex and tRNA, when tested with various antigens. None of the sera tested gave a precipitate with tRNA alone. Antisera from animals immunized with either tRNA-MBSA complex or tRNA alone failed to precipitate with tRNA-BSA or tRNA-(T, G)-A--L conjugates. On the other hand, both the rabbit antisera against tRNA-BSA and the goat antiserum against tRNA-(T, G)-A--L

Table I

Precipitation of antisera to tRNA with various antigens

Immunogen	Testing antigen			
	BSA	tRNA	tRNA-BSA	tRNA-(T, G)-A--L (precipitating antibodies ^a)
tRNA-BSA	4/4	0/4	4/4	4/4
tRNA-(T, G)-A--L ^b	0/1	0/1	1/1	1/1
tRNA and MBSA	2/4	0/4	2/4	0/4
tRNA	0/4	0/4	0/4	0/4
BSA	4/4	0/4	4/4	0/4
(T, G)-A--L	0/2	0/2	0/2	2/2

^aExpressed as the ratio of animals producing precipitating antibodies to the total number of animals.

^bThis conjugate was used to immunize a goat. All the others were injected into rabbits.

precipitating anti-tRNA antibodies. Thus, precipitating antibodies to tRNA could be obtained only following immunization with tRNA covalently bound to a protein or a synthetic polypeptide.

Antibodies against tRNA could be selectively absorbed from the antisera with tRNA-Sepharose or tRNA-(T, G)-A--L-Sepharose. This absorption could be applied for the preparation of purified antibodies to tRNA.

Specific antibodies to tRNA can be demonstrated by the binding of ³²P-tRNA to antibody-Sepharose columns. These columns were prepared by coupling to Sepharose the gamma globulin fraction from the different antisera. As a control, the gamma globulin fraction from an unrelated anti-lysozyme serum was similarly coupled. The antibody-Sepharose columns prepared from antisera to tRNA-BSA were able to bind ³²P-tRNA (Fig. 3), whereas columns prepared from the other sera did not bind any ³²P-tRNA and behaved like the control anti-lysozyme-Sepharose column. About 20% of the ³²P-tRNA applied was retained on the anti-tRNA-BSA-Sepharose column. This labelled tRNA could be specifically displaced by unlabelled tRNA, as is shown in Fig. 3. The displaced ³²P-tRNA was found to precipitate with 5% trichloroacetic acid, suggesting that the bound material was free of small oligonucleotides.

The results reported here demonstrate that antibodies to tRNA may be obtained upon immunization with covalently bound tRNA conjugates. We could not demonstrate

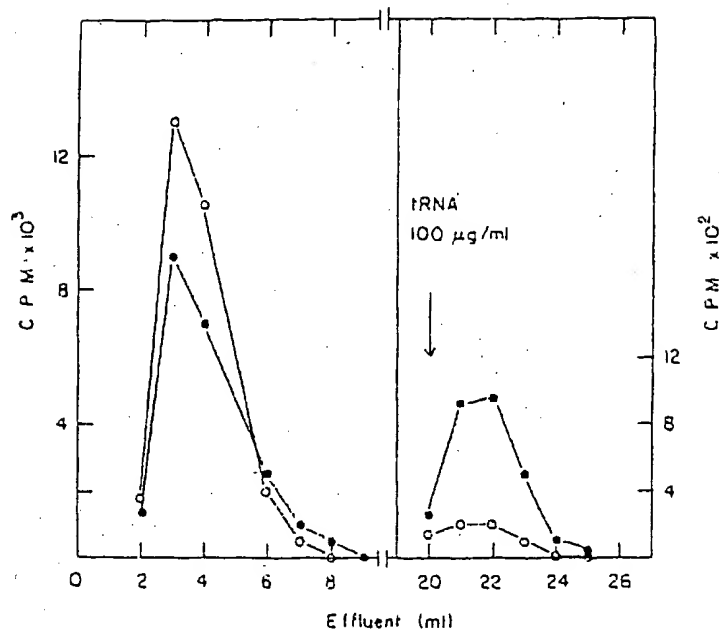


Fig. 3: Binding of ^{32}P -tRNA (*E. coli*) to specific antibodies coupled to Sepharose. ^{32}P -tRNA solution (40,000 cpm) was added to antibody-Sepharose columns (3 x 0.3 cm) prepared as described in the text, and left for 15 minutes at room temperature. The columns were then washed with PBS until no further radioactive material came through. The collected effluents were counted for radioactivity. A solution of unlabeled *E. coli* tRNA (100 $\mu\text{g}/\text{ml}$) was then added to the column and the displaced radioactive ^{32}P -tRNA was collected and counted. ●, antibody-Sepharose column prepared with gamma globulin from antiserum to tRNA-BSA; ○, antibody-Sepharose columns prepared with gamma globulin from antiserum, to tRNA, a complex of tRNA and MBSA and hen egg-white lysozym

the presence of antibodies to tRNA following immunization with either tRNA alone or electrostatic complex of tRNA and MBSA.

The conjugation of tRNA to a carrier with EDCI, which probably takes place through one phosphate residue of tRNA and the amino groups of the carrier, has the advantage that the conjugate may keep the tRNA in its native conformation. (In amino acid charging experiments with the conjugates show that the covalently bound is at least as active as free tRNA). Therefore, the production of specific anti-tRNA by such conjugates may be useful in structural studies of tRNA. Thus, a tRNA may be directed against different antigenic determinants derived from different regions, as well as conformational areas of the molecule. Some of the antibodies produced may recognize even unique nucleotide residues. Indeed, anti-tRNA could inactivate inosine-bacteriophage T4 (12).

REFERENCES

1. Plescia, O. J. and Braun, W., *Adv. Immunol.* **6**, 231 (1967).
2. Levine, L. and Stollar, B. D., *Progress in Allergy* **12**, 161 (1968).
3. Bigley, N. J., Dodd, M. C. and Geyer, V. B., *J. Immunol.* **90**, 416 (1963).
4. Hernandez, R., Burgin-Wolff, A. and Just, M., *Eur. J. Biochem.* **6**, 23 (1968).
5. Plescia, O. J., Palczuk, N. C., Cora-Figueroa, E., Mukherjee, A. and Braun, W., *Proc. Nat. Acad. Sci.* **54**, 1281 (1965).
6. Zimmerman, S. B. and Sandeen, G., *Analytical Biochem.* **14**, 269 (1966).
7. Lowry, O. H., Rosenbrough, N. J., Farr, A. L. and Randall, R. J., *J. Biol. Chem.* **193**, 265 (1951).
8. Fuchs, S. and Sela, M., *Biochem. J.* **87**, 70 (1963).
9. Porath, J., Axen, R. and Ernback, S., *Nature* **215**, 1491 (1967).
10. Givol, D., Weinstein, Y., Gorecki, M. and Wilchek, M., *Biochem. Biophys. Res. Comm.* **33**, 825 (1970).
11. Halloran, M. J. and Parker, C. W., *J. Immunol.* **96**, 373 (1966).
12. Bonavida, B., Fuchs, S., Inouye, H., and Sela, M., unpublished results.

the

only

ERNA

s to

dies

ntial

dies

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s): Engelhardt et al.

Serial No.: 08/479,997

Filed: June 7, 1995

For: OLIGO- OR POLYNUCLEOTIDES COMPRISING
PHOSPHATE MOIETY LABELED NUCLEOTIDES
(As Previously Amended)

Group Art Unit: 1656

Examiner: Alexander H. Spiegler

527 Madison Avenue, 9th Floor
New York, New York 10022
May 28, 2002

FILED VIA EXPRESS MAIL

Honorable Commissioner of Patents and Trademarks
Washington, D.C. 20231

**THIRD SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT
UNDER 37 C.F.R. §§1.56 & 1.97-1.98**

Dear Sirs:

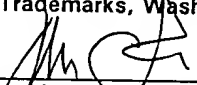
Pursuant to the provisions of 37 C.F.R. §§1.97-1.98, and in full compliance with their duty of disclosure under 37 C.F.R. §1.56, Applicants, through their attorney, are bringing the following twenty-nine (29) documents to the attention of the U.S. Patent and Trademark Office and the Examiner handling their above-identified application:

Dean L. Engelhardt et al.

Serial No.: 08/479,997

Filed: June 7, 1995

Page 2 [Third Supplemental IDS -- May 28, 2002]

EXPRESS MAIL CERTIFICATE	
"Express Mail" Label No.	<u>EL839968769US</u>
Deposit Date	<u>May 28, 2000</u>
I hereby certify that this paper and the attachments herein are being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Commissioner of Patents and Trademarks, Washington DC 20231.	
 _____ Ronald C. Fedus Reg. No. 32,567	<u>MAY 28 2002</u> _____ Date

1. Pollack, S. E. and Auld, D. S., "Fluorescent Nucleotide Triphosphate Substrates for Snake Venom and Phosphodiesterase," Analytical Biochemistry 127: 81-88 (1982) [Exhibit 1];
2. Scopes, D. I. C., et al., "Defined Dimensional Changes in Enzyme Cofactors: Fluorescent "Stretched-Out" Analogs of Adenine Nucleotides," Science 195: 296-298 (1977) [Exhibit 2];
3. Onodera, M. and Yagi, K., "Synthesis of 2-(Dansylamino)Ethyl Triphosphate and its Properties as a Fluorescent Substrate of Heavy Meromyosin-ATPase," Biochimica Et Biophysica 253: 254-265 (1971) [Exhibit 3];
4. Yarbrough, L. R., "Synthesis and Properties of a New Fluorescent Analog of ATP: Adenosine-5'-Triphospho- γ -1-(5-Sulfonic Acid) Naphthylamidate," Biochemical and Biophysical Research Communications 81(1): 35-41 (1978) [Exhibit 4];
5. Silver, M. S. and Fersht, A. R., "Direct Observation of Complexes Formed between recA Protein and a Fluorescent Single-Stranded Deoxyribonucleic Acid Derivative," Biochemistry 21: 6066-6072 (Received April 16, 1982) [Exhibit 5];
6. Crane, L. J. and Miller, D. L., "Guanosine Triphosphate and Guanosine Diphosphate as Conformation-Determining Molecules. Differential Interaction of a Fluorescent Probe with the Guanosine Nucleotide Complexes of Bacterial Elongation Factor Tu," Biochemistry 13(5): 933-938 (1974) [Exhibit 6];
7. Hertz, H. S. and Zachau, H. G., "Kinetic Properties of Phenylalanyl-tRNA and Seryl-tRNA Synthetases for Normal Substrates and Fluorescent Analogs," Eur. Journal of Biochemistry 37: 203-213 (1973) [Exhibit 7];
8. Wu, F. Y. H. and Wu, C. W., "Fluorescent Affinity Labeling of Initiation Site on Ribonucleic Acid Polymerase of *Escherichia coli*," Biochemistry 13(12): 2562-2566 (1974) [Exhibit 8];
9. McKenzie, R. L., et al., "Fluorescent Conjugates of Natural and Biosynthetic Polynucleotides," Biochimica Et Biophysica ACTA 277: 306-322 (1972) [Exhibit 9];
10. Tournon, J., "Fluorescence Probing of Nucleic Acids: I. singly and doubly labeled dithymidine phosphate: fluorescence and energy transfer studies," Nucleic Acids Research 2(8): 1261-1273 (1975) [Exhibit 10];

Dean L. Engelhardt et al.

Serial No.: 08/479,997

Filed: June 7, 1995

Page 4 [Third Supplemental IDS -- May 28, 2002]

11. Erlanger, B. F., et al., "Use of Antibodies to Nucleosides and Nucleotides in Studies of Nucleic Acids in Cells," Methods in Enzymology 60: 302-307 (1975) [Exhibit 11];
12. Yarbrough, L. R., et al., "Synthesis and Properties of Fluorescent Nucleotide Substrates for DNA-dependent RNA Polymerases," The Journal of Biological Chemistry 254(23): 12069-12073 (1979) [Exhibit 12];
13. Wintermeyer, W. and Zachau, H. G., "Fluorescent Derivatives of Yeast tRNA^{Phe}," Eur. Journal of Biochem 98: 465-475 (1979) [Exhibit 13];
14. Reines, S. A. and Schulman, L. H., "A New Method for Attachment of Fluorescent Probes to tRNA," Methods in Enzymology 61: 146-157 (1979) [Exhibit 14];
15. Barrio, J. R., et al., "Synthesis of Modified Nucleoside 3', 5'-Bisphosphates and Their Incorporation into Oligoribonucleotides with T4 RNA Ligase," Biochemistry 17(11): 2077-2081 (1978) [Exhibit 15];
16. Koenig, P., et al., "Pyrene Derivatives as Fluorescent Probes of Conformation Near the 3' Termini of Polyribonucleotides," Biopolymers 16: 2231-2242 (1977) [Exhibit 16];
17. Menzel, H. M., "On the Phe-tRNA induced binding of fluorescent oligonucleotides to the ribosomal decoding site," Nucleic Acids Research 4(8): 2881-2892 (1977) [Exhibit 17];
18. Leonard, N. J. and Tolman, G. L., "Fluorescent Nucleosides and Nucleotides," Annals of the New York Academy of Sciences 255: 43-58 (1975) [Exhibit 18];
19. Gavrilovskaya, I. N., et al., "Immunofluorescent Demonstration of Double-Stranded RNA and Virus Antigen in RNA Virus-Infected Cells," Virology 62: 276-279 (1974) [Exhibit 19];
20. Kukhanova, M. K., et al., "Peptidyl-tRNA With A Fluorescent Label: Ribosome Substrates in Peptide Bond Formation," Molecular Biology Reports 1: 397-400 (1974) [Exhibit 20];
21. Lynch, D. C. and Schimmel, P. R., "Effects of Abnormal Base Ionizations on Mg²⁺ Binding to Transfer Ribonucleic Acid as Studied by a Fluorescent Probe," Biochemistry 13(9): 1852-1861 (1974) [Exhibit 21];

Dean L. Engelhardt et al.

Serial No.: 08/479,997

Filed: June 7, 1995

Page 5 [Third Supplemental IDS -- May 28, 2002]

22. Bergstrom, D. E. and Leonard N. J., "Structure of the Borohydride Reduction Product of Photolinked 4-Thiouracil and Cytosine. Fluorescent Probe of Transfer Ribonucleic Acid Tertiary Structure," Journal of the American Chemical Society 94(17): 6178-6182 (1972) [Exhibit 22];
23. Mundry, K. W. and Priess, H., "A Quantitative Technique for Mapping Oligonucleotides on Thin Layers of Cellulose," Biochimica Et Biophysica ACTA 269: 225-236 (1972) [Exhibit 23];
24. Munniger, K. O. and Chang, S. H., "A Fluorescent Nucleoside from Glutamic Acid tRNA of *Escherichia coli* K12," Biochemical and Biophysical Research Communications 46(5): 1837-1842 (1972) [Exhibit 24];
25. Erlanger, B. F., et al., "Nucleic Acid-Reactive Antibodies Specific for Nucleosides and Nucleotides," Acta endocrinologica 71: 206-221 (1972) [Exhibit 25];
26. Yoshikami, D. and Keller, E. B., "Chemical Modification of the Fluorescent Base in Phenylalanine Transfer Ribonucleic Acid," Biochemistry 10(15): 2969-2976 (1971) [Exhibit 26];
27. Rudkin G. T. and Siollar, B. D., "High Resolution Detection of DNA-RNA Hybrids *in situ* by Indirect Immunofluorescence," Nature 265: 472-473 (1977) [Exhibit 27];
28. Rozovskaya, T. A., et al., "Introduction of a Fluorescent Label at the 3' -OH End of DNA and the 3' -OH End of the Growing RNA Chain," Molekulyama Biologiya 11(3): 598-610 (1977) [Exhibit 28]; and
29. Kossel, H. and Seliger, H., "Recent Advances in Polynucleotide Synthesis," Progress in the Chemistry of Organic Natural Products 32: 297-508 (1975) [Exhibit 29].

The above documents [Exhibits 1-29] were brought to the attention of Applicants' attorney earlier this month by a third party.

A completed Form PTO-1449 listing the 29 above-submitted documents is also attached hereto as Exhibit 30.

By this voluntary citation of art, Applicants and their attorney are requesting that the documents be made of record in the present application.

The above citation of documents is not a representation that these documents constitute a complete or exhaustive listing, nor that the above listing necessarily includes the closest or most relevant documents, nor are these documents necessarily a complete listing of all documents known to Applicants or their attorney. It is simply a voluntary citation of documents made in good faith, which is not intended to serve in any way as a substitute for the Examiner's own search.

In view of the general and specific features described and claimed in the present application, Applicants respectfully submit that the present invention is neither disclosed nor suggested by the documents referred to above and is thus patentably distinct thereover. Furthermore, Applicants do not believe, and do not submit, by the citation of these references, that these documents, either by themselves or in combination with other documents, render the invention *prima facie* obvious under the duty of disclosure rules.

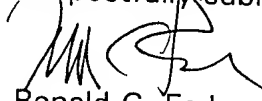
Applicants respectfully request that the Examiner make the above-submitted documents of record in the instant application. Applicants further request that the Examiner consider these documents as any of them may relate to the instant application.

The fee under 37 C.F.R. §1.17(p) for filing this Supplemental Information Disclosure Statement is \$180.00. The Patent and Trademark Office is hereby

Dean L. Engelhardt et al.
Serial No.: 08/479,997
Filed: June 7, 1995
Page 7 [Third Supplemental IDS -- May 28, 2002]

authorized to charge the amount of this fee (and any other fees in connection with this IDS) to Deposit Account No. 05-1135, or to credit any overpayment thereto.

Respectfully submitted,



Ronald C. Fedus
Registration No. 32,567
Attorney for Applicants

ENZO LIFE SCIENCES, INC.
(formerly ENZO DIAGNOSTICS, INC.)
c/o Enzo Biochem, Inc.
527 Madison Avenue, 9th Floor
New York, New York 10022
Tel. (212) 583-0100

* * * * *

Ig\uspros\Enz5D6C2.3rdIDS

ie, K., and Grossman, L., eds.),
15-511, Academic Press, New

imura, H., and Nishizuka, Y.
Biophys. Res. Commun. 41,

y, S. L., and Park, C. R. (1975)
50, 218-225.

alsh, D. A. (1971) *Biochemistry*

erin, W., Kubler, D., and Kinzel,
Biochem. 93, 52-59.

en, O. M. (1974) *Anal. Biochem.*

hmed, O., Marchis-Mouren, G.,
(1982) *Biochem. Biophys. Acta*

Fluorescent Nucleotide Triphosphate Substrates for Snake Venom Phosphodiesterase¹

STUART E. POLLACK² AND DAVID S. AULD³

Center for Biochemical and Biophysical Sciences and Medicine, Harvard Medical School, and the Division of Medical Biology, Brigham and Women's Hospital, Boston, Massachusetts

Received May 17, 1982

The fluorophore 1-aminonaphthalene-5-sulfonate has been coupled to the γ -phosphorus of dTTP, CTP, UTP, ATP, GTP, dCTP, and dGTP via a phosphoramidate linkage. The synthetic reaction was monitored by chromatography on an analytical μ Bonadpak HPLC column. Changes in the fluorescence of these nucleotide analogs upon cleavage of the α - β phosphodiester bond provide a sensitive and convenient steady-state assay of enzymes which act upon this bond and are active within the pH range of 4 to 10. For pyrimidine derivatives this cleavage increases fluorescence above 350 nm 2- to 6-fold while for purines it quenches it 1.2- to 1.4-fold. The kinetic constants determined by initial rate measurements and from integrated Michaelis-Menten equations indicate that these fluorescent nucleotides are excellent substrates of snake venom phosphodiesterase from *C. adamanteus*. The fluorophoric group does not interfere with binding or catalysis. This is consistent with product inhibition studies that demonstrate that most of the substrate binding strength resides in the nucleotide monophosphate moiety. The substrates bind tightly with K_m 's ranging from 5 to 30 μ M and are hydrolyzed rapidly. Values of k_{cat} range from 200 to 600 s^{-1} at pH 8.0 and 20°C. A wide variety of fluorophores are therefore possible without greatly affecting the catalysis of the substrate by snake venom phosphodiesterase.

Fluorescent *N*-dansylated peptides have been very useful for mechanistic studies of proteolytic enzymes (1, and references therein). The introduction of the dansyl fluorophore into peptides which have a C-terminal tryptophanyl residue leads to intramolecular radiationless energy transfer between the donor tryptophan and the acceptor dansyl group, resulting in nearly complete quenching of tryptophan fluorescence. On cleavage of the bond adjacent to the C-ter-

minus by carboxypeptidase A there is a 100-fold increase in tryptophan fluorescence which provides a direct and rapid initial rate assay for the enzyme (2).

Measurement of intermolecular radiationless energy transfer (RET)⁴ between carboxypeptidase tryptophanyl residues and the dansyl group in the substrate by stopped-flow fluorescence allows direct observation of the ES complex (3). We have applied this approach to a wide variety of proteolytic enzymes and a detailed theoretical description of the RET kinetic analysis at both steady

¹ This work was supported by Grant-in-Aid GM-24968 from the National Institutes of Health of the Department of Health, Education and Welfare to Harvard Medical School.

² National Institutes of Health Fellow supported by National Research Service Award 1 F32 HL06050-01 from the National Heart, Lung and Blood Institute.

³ To whom all correspondence should be addressed: Division of Medical Biology, Brigham and Women's Hospital, Seeley G. Mudd Bldg./250, Longwood Ave., Boston, Mass. 02115.

⁴ Abbreviations used: 1,5-ANS, 1-aminonaphthalene-5-sulfonate (the nomenclature chosen is that suggested by Weber (8)); NTP, NDP, and NMP, nucleotide tri-, di-, and monophosphates, respectively; TEA, triethylammonium bicarbonate; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; RET, radiationless energy transfer; ES complex, enzyme-substrate complex.

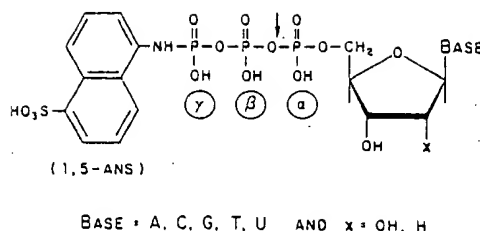


FIG. 1. Structure of the nucleotide triphosphate analogs. The arrow shows the point of cleavage by snake venom phosphodiesterase.

state and pre-steady state has been presented (1,4,5). The RET kinetic approach can be applied to any enzyme whose substrate can be labeled with a suitable fluorophore, providing the probe does not drastically affect binding or catalysis. The application of these fluorescent techniques to nucleic acid metabolizing enzymes would be of great value since they are usually assayed by tedious radioactive means.

Snake venom phosphodiesterase, which catalyzes the hydrolysis of an α - β phosphodiester bond of nucleotide triphosphates, was chosen for the initial studies. This enzyme has mechanistic features similar to those of RNA and DNA polymerases in that it formally transfers a nucleotide monophosphate group to water while the polymerases which also act at the α - β phosphodiester bond of nucleotide triphosphates transfer the nucleotide monophosphate group to a growing polynucleotide chain. In addition snake venom phosphodiesterase from *C. adamantanus* like all known RNA and DNA polymerases (6,7) is a zinc metalloenzyme (Pollack and Auld, in preparation).

We report herein the synthesis of fluorescent 1-aminonaphthalene-5-sulfonic acid (1,5-ANS) derivatives of nucleotide triphosphates (Fig. 1) and the design of a fluorescent assay for their hydrolysis by snake venom phosphodiesterase. A preliminary account of this work has been presented (9).

MATERIAL AND METHODS

Snake venom phosphodiesterase was obtained as a lyophilized powder from Wor-

thington Biochemical Corporation. It had been prepared by the procedure of Williams *et al.* (10) and treated to inactivate 5'-nucleotidase activity by incubation at 37°C, pH 3.6, for 3 h (11). The enzyme was further purified by blue Sepharose chromatography (Pollack and Auld, in preparation).

All nucleotide triphosphates and Tris were purchased from Sigma Chemical Company. Protein concentration was determined by the Bio-Rad assay procedure. Measurements were made in triplicate and average values were used. Protein assay reagent and protein standard bovine plasma γ -globulin were obtained from Bio-Rad Laboratories.

Absorption and fluorescent spectra were recorded on a Cary 219 and a Perkin-Elmer MPF-2A spectrophotometer, respectively. Fluorescent spectra were always obtained on samples that had an absorbance of 0.1 or less to prevent significant inner filter effects. Peak areas were computed using a Hewlett-Packard Model 9810A calculator, or an Apple II⁺ microcomputer. pH was measured with a Markson model 90 pH meter and conductivity with a Radiometer conductivity meter. High-performance liquid chromatography was done using an ALC 200 chromatograph (Waters Associates, Milford, Mass.) equipped with a Model 660 solvent programmer and a Model 440 absorbance detector, which measured OD at 254 nm. An analytical (30 \times 3.9 mm) C₁₈ μ Bondapak column (Waters Associates) was used for nucleotide separation.

Synthesis of fluorescent nucleotides.⁵ The 1,5-ANS (ICN Laboratories) was recrystallized from a 10% ethanol-water mixture which was treated with activated charcoal (Norite) and filtered through Hyflo Super Cel (Fisher Scientific) to remove the charcoal. In a typical reaction, the sodium salt of a nucleotide triphosphate, NTP, 120 mg, is added

⁵ γ -Substituted ATP derivatives have also been prepared under nonaqueous conditions (28). The acid-catalyzed activation of ATP with DCC results in the formation of adenosine 5'-trimetaphosphate which upon addition of an amine yields the γ -substituted ATP derivative.

mical Corporation. It had the procedure of Williams treated to inactivate 5'-nucleotides by incubation at 37°C, pH 7.5. The enzyme was further purified by Sepharose chromatography (d, in preparation). The triphosphates and Tris were from Sigma Chemical Company. The reaction was determined by the procedure. Measurements were made and average values were calculated. Reagent and protein standards and γ -globulin were obtained from various laboratories.

and fluorescent spectra were recorded on a Cary 219 and a Perkin-Elmer fluorescence photometer, respectively. Spectra were always obtained on a quartz cell with an absorbance of 0.1 or less to avoid inner filter effects. Peak intensities were measured using a Hewlett-Packard calculator, or an Apple II+ computer. pH was measured with a Beckman 90 pH meter and conductivity with a Beckman liquid chromatography conductivity meter. The liquid chromatography system (ALC 200 chromatograph, Waters, Milford, Mass.) equipped with a solvent programmer and an absorbance detector, which monitored at 254 nm. An analytical (30 μ m) μ Bondapak column (Waters) was used for nucleotide separation.

fluorescent nucleotides.⁵ The nucleotides (Laboratories) was recrystallized from 90% ethanol-water mixture and activated charcoal removed through Hyflo Super Cel (Whatman) to remove the charcoal. In the reaction, the sodium salt of a nucleotide, NTP, 120 mg, is added

to 19 ml of water at $20 \pm 2^\circ\text{C}$ and the pH is adjusted to 6.0. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide, EDC (500 mg, Sigma Chemical Co.) is then added over 3 min and the pH is kept in the range 5.6 to 5.9 with the addition of small aliquots of 0.4 N HCl. After 10 min, 3.4 ml of a 0.6 M 1,5-ANS solution at pH 6.5 was added. The reaction mixture is allowed to stand for 18 h at room temperature and is then diluted with distilled H_2O until the ionic strength is equal to that of 0.09 M TEA buffer and applied to a DEAE-cellulose (Whatman) column (1.5 \times 16.0 cm) equilibrated with 0.09 M TEA buffer, pH 7.5, and eluted stepwise with increasing TEA buffer concentrations. The 0.09 M TEA buffer elutes unreacted 1,5-ANS. A 0.16 M TEA buffer elution removes the unreacted NTP and a side product, which has the same physical properties as the 1,5-ANS-NTP except retention time on the HPLC column. The 0.28 M TEA buffer elutes the 1,5-ANS-NTP product. Purity of the preparations is assessed by HPLC. The fractions containing product are pooled and lyophilized. The solid is redissolved in a small amount of water and again lyophilized, as necessary to remove excess TEA buffer. Yields of 55–65% are obtained.

Enzyme assays. Snake venom phosphodiesterase is assayed in 50 mM Tris, pH 8.0, containing 15 mM MgCl_2 at 20°C . Enzymatic activity is measured by observing the change in fluorescence above 410 nm which occurs upon hydrolysis of 1,5-ANS-NTP. Excitation of the substrate is at 320 nm. Kinetic parameters were determined from Lineweaver-Burk or Woolf plots, keeping the substrate concentration within the range 10 to 0.1 K_m .

Stopped-flow fluorescence measurements were made on a stopped-flow instrument equipped with quartz fiber optics, a Schoeffel monochromator, a 200-W Xenon lamp, and a low noise/high sensitivity detector system comprised of a 9526B trialkyl (S13) photomultiplier and a Sorenson high-voltage power supply (12). The time course of the fluorescence changes were stored digitally on floppy disks (Memorex) using a DEC PDP 11/34

computer, equipped with an AR11 A/D converter and a VT-55 Decscope, and a Decwriter III printer. Equal volumes (0.2 ml) of nanomolar enzyme and micromolar substrate solutions were mixed for each progress curve or initial rate analysis. The complete conversion of substrate to product for each experiment was recorded as 1000 data points at equal time intervals for progress curve analysis. Parameters in an integrated Michaelis-Menten treatment of the data were routinely computed by unweighted nonlinear regression analysis with data points typically beginning at 10% of reaction and in all cases extending to 90% of reaction. The time course of the reaction that is predicted from the calculated Michaelis-Menten parameters is superimposed on the observed data on the VT-55 screen for visual inspection and the values of V_{\max} and K_m and their associated error analysis are automatically recorded on a floppy disk.

Initial rate measurements were determined from at least 300 data points, collected over the first 10% of the reaction. First-order rate constants were obtained by linear regression of $-\log(F_\infty - F_t)$ versus time. The first-order plot was examined and the results were stored as described above. Each data point on a kinetic plot is the average of two to four determinations.

RESULTS

The coupling of 1,5-ANS to NTP was optimized by measuring the extent of coupling as a function of the concentration of reactants, pH, temperature, and time of mixing. Aliquots of the reaction mixture were injected onto an analytical C_{18} μ Bondapak HPLC column. Isocratic elution with 25 mM ammonium phosphate buffer, pH 8.0, allowed the reactants, e.g., ATP and 1,5-ANS, and the products 1,5-ANS-ATP, ADP, and AMP to be separated at a pressure of 550 psi (Table 1). The results for TTP are given for comparison. The rate and extent of the coupling reaction can be measured by monitoring the HPLC elution profile as a function

TABLE I
HPLC RETENTION TIME OF REACTION MIXTURE
COMPONENTS AND NUCLEOTIDES^a

Compound	Retention time (min)
1,5-ANS-ATP (1,5-ANS-TTP)	3.97 (3.76)
1,5-ANS	9.39
ATP (TTP)	4.40 (4.35)
ADP (TDP)	4.80 (4.63)
AMP (TMP)	4.98 (5.47)

^a Isocratic elution with 25 mM ammonium phosphate, pH 8.0, at a flow rate of 0.8 ml/min (550 psi) on a C₁₈ μ Bondapak column.

of time. A decrease occurs in the ATP peak with a concomitant increase of the product 1,5-ANS-ATP peak. It was also possible to collect sufficient amounts of products from HPLC for measurement of their absorption and fluorescence spectra. The fractions eluted from the DEAE-cellulose column could thus be identified by their HPLC retention times as well as their absorption and fluorescence spectra. Using molar absorptivities of 6100 at 330 nm for 1,5-ANS (8) and 15,400 for ATP at 260 nm (13), the absorption spectra of 1,5-ANS-ATP indicated a 1:1 stoichiometry for the two constituents.

Hydrolysis of 1,5-ANS-ATP by snake venom phosphodiesterase was followed as a function of time. Aliquots of the hydrolysis mixture were passed through Millipore filters (0.6 μ m) to remove enzyme and then injected onto the μ Bondapak column. The 1,5-ANS-ATP peak decreased concomitantly with an increase in the AMP and 1,5-ANS-pyrophosphate peaks. No intermediate ADP nor any ATP was ever seen in these hydrolysis mixtures. Hence, the point of enzymatic cleavage is between the α - β phosphoryl bond of the NTP (Fig. 1).

Excitation of the purines 1,5-ANS-ATP, 1,5-ANS-dGTP, 1,5-ANS-GTP and the pyrimidines 1,5-ANS-dCTP, 1,5-ANS-CTP, 1,5-ANS-UTP, and 1,5-ANS-TTP at 320 nm leads to emission of maximum fluores-

cence at approximately 444 nm under assay conditions of 0.015 M MgCl₂, 0.05 M Tris, pH 8.0 at 25°C. Cleavage of the α - β phosphoryl bond shifts the fluorescence maximum to 456 nm and enhances fluorescence above 350 nm 2- to 6-fold for pyrimidines but quenches fluorescence 1.2- to 1.4-fold for purines. These spectral differences are shown in Fig. 2 for 1,5-ANS-dGTP, 1,5-ANS-dTTP, and their hydrolysis product 1,5-ANS-pyrophosphate. The different fluorescence properties of 1,5-ANS-pyrophosphate and 1,5-ANS-NTP can be used to design a steady-state assay.

The useful pH range of this fluorescent assay was investigated by measuring the total fluorescence above 350 nm for 1,5-ANS-TTP and its hydrolysis products 1,5-ANS-pyrophosphate and TMP at different pH values (Table 2). The large increase in fluorescence observed over the entire pH range indicates it should form the basis of a useful assay for any enzyme acting on the α - β phosphoryl bond and having a pH optimum between 4 and 10.

The increase in fluorescence on hydrolysis of the pyrimidine substrate 1,5-ANS-TTP, 4 μ M, catalyzed by snake venom phosphodiesterase, 2.2×10^{-8} M, is shown in Fig. 3. Since the data can be collected over different

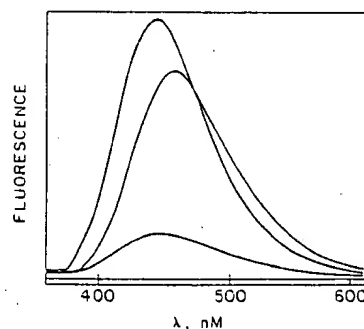


FIG. 2. Fluorescence spectra of 1,5-ANS-TTP (lowest curve), 1,5-ANS-dGTP (highest curve), and 1,5-ANS-pyrophosphate (middle curve). Catalytic amounts of snake venom phosphodiesterase, in 0.015 M MgCl₂, 0.05 M Tris, pH 8.0, were used to generate the 1,5-ANS-pyrophosphate derivative. Excitation is at 320 nm and emission is observed using a 350-nm cutoff filter.

TABLE 2

 TOTAL INCREASE IN FLUORESCENCE ABOVE 350 nm
 FOR THE HYDROLYSIS OF 1,5-ANS-TTP BY SNAKE
 VENOM PHOSPHODIESTERASE AT DIFFERENT pH'S^a

pH	F_p/F_s
4.00	5.7
4.55	4.9
5.10	5.4
5.50	6.2
6.10	6.3
7.00	6.3
8.00	5.4
9.00	4.9
10.00	2.7

^a Excitation is at 320 nm using a 350-nm cutoff filter. The fluorescence of 2×10^{-6} M 1,5-ANS-TTP, F_s , or 1,5-ANS pyrophosphate, F_p , is measured in 0.015 M MgCl₂, 0.05 M Tris.

time ranges and digitally stored, several hundred points are available for both initial rate and progress curve analysis. For the example shown, 500 data points were collected over two consecutive time intervals of 0.8 and 13 s. (Fig. 3). The first time interval is used for initial rate measurements while both time intervals can be used for progress curve analysis. Measurement of initial rates of 1,5-ANS-TTP hydrolysis yields a value of 36.9 μ M for K_m and 590 s⁻¹ for k_{cat} , calculated from a Lineweaver-Burk plot (14) (Fig. 4).

The time course of the reaction at each substrate concentration was also analyzed with an integrated Michaelis-Menten equation, which takes into account competitive product inhibition by NMP,

$$\frac{t}{\ln(S/S_i)} = \frac{1}{V_{max}} (1 - K_m/K_p) \frac{S - S_i}{\ln(S/S_i)} + \frac{K_m}{V_{max}} (1 + S/K_p), \quad [1]$$

where S is the initial substrate concentration and that at time t is S_i .

The slope and intercept of this equation are obtained from unweighted nonlinear regression of $t/\ln(S/S_i)$ versus $(S - S_i)/$

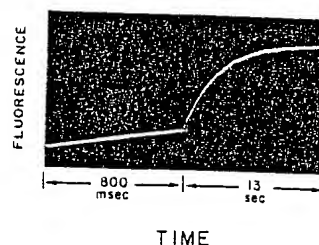


FIG. 3. Stopped-flow fluorescence observation of the hydrolysis of 1,5-ANS-TTP, 4 μ M, by purified snake venom phosphodiesterase, 2.2×10^{-8} M, in 0.015 M MgCl₂, 0.05 M Tris, pH 8.0, at 20°C. Excitation is at 320 nm, and emission is observed with a 410-nm cutoff filter. Five hundred data points are collected for both the 0.8- and 13-s time intervals during which hydrolysis occurs.

$\ln(S/S_i)$. The kinetic constants K_m , k_{cat} , and K_p cannot be determined from a single straight line, but they can be found readily from a series of experiments with different S values. Jennings and Niemann (15) have pointed out that Eq. [1] is of the form

$$\frac{S}{V} = \frac{S}{V_{max}} (1 - K_m/K_p) + \frac{K_m}{V_{max}} (1 + S/K_p). \quad [2]$$

Equation [2] can be further simplified to that of a Woolf plot (16),

$$E_T S/V = (S + K_m)/k_{cat}, \quad [3]$$

by multiplying the slope, $V_{max}(1 - K_m/K_p)$,

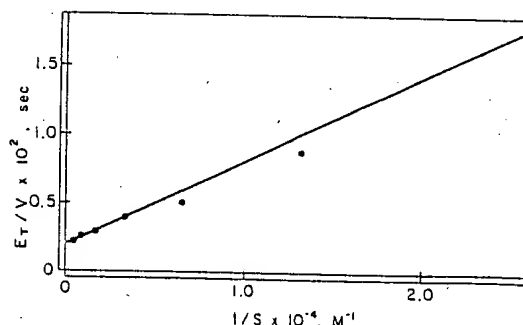
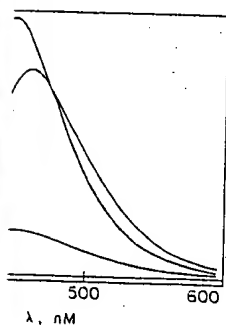


FIG. 4. Initial rate determination of the kinetic parameters K_m and k_{cat} for the hydrolysis of 1,5-ANS-TTP by snake venom phosphodiesterase in 0.015 M MgCl₂, 0.05 M Tris, pH 8.0, at 20°C.

ately 444 nm under assay 5 M MgCl₂, 0.05 M Tris, cleavage of the α - β phospho- the fluorescence maximum enhances fluorescence 6-fold for pyrimidines 1.2- to 1.4-fold for differences are shown 1,5-ANS-dGTP, 1,5-ANS- hydrolysis product 1,5-ANS- the different fluorescence 1,5-ANS-pyrophosphate and be used to design a

range of this fluorescent ed by measuring the total 350 nm for 1,5-ANS- hydrolysis products 1,5-ANS- TMP at different pH val- large increase in fluores- the entire pH range in- form the basis of a useful ie acting on the α - β phos- having a pH optimum be-

fluorescence on hydrolysis substrate 1,5-ANS-TTP, snake venom phospho-)⁻⁸ M, is shown in Fig. 3. be collected over different



pectra of 1,5-ANS-TTP (lowest curve), and 1,5-ANS- (highest curve). Catalytic amounts of esterase, in 0.015 M MgCl₂, 0.05 M Tris, pH 8.0, at 20°C. Excitation is at 320 nm and emission is observed with a 350-nm cutoff filter.

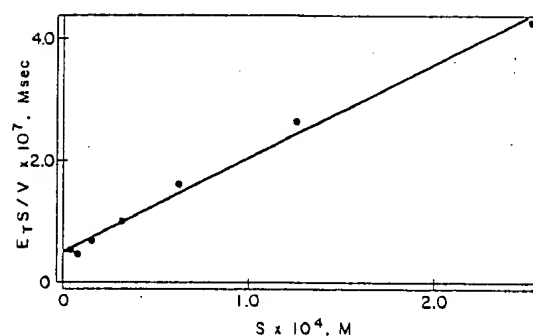


FIG. 5. Determination of the kinetic parameters K_m and k_{cat} for the hydrolysis of 1,5-ANS-TTP by progress curve analysis (see Eqs. [1]–[3]).

by the substrate concentration, and adding it to the intercept $K_m/V_{max}(1 + S/K_p)$, where

$$k_{cat} = V_{max}/E_T.$$

This mathematical method is equivalent to an extrapolation to the initial part of the reaction to time $t = 0$, where no inhibition by product can occur. Hence, terms in K_p drop out of Eq. [2] to yield Eq. [3]. These S/V points when plotted as a Woolf plot versus S allow one to solve for the true values of K_m and k_{cat} . Hydrolysis of 1,5-ANS-TTP by purified snake venom phosphodiesterase yields a K_m of $30.3 \mu\text{M}$ and a k_{cat} of 500 s^{-1} (Fig. 5). The parameters agree well with those determined by initial rate measurements (see below).

The inhibitor constant K_p for the product TMP, obtained from a plot of the intercepts of Eq. [1], $(K_m/k_{cat})(1 + S/K_p)$ versus initial substrate concentration, is $38 \mu\text{M}$ (Fig. 6). This value agrees favorably with that of $24 \mu\text{M}$ obtained by addition of TMP to the assay.

The kinetic parameters, K_m , k_{cat} , and K_p for a number of ribo- and deoxypurine and pyrimidine analogs are given in Table 3. The commercial preparation of the enzyme was used for these studies.

DISCUSSION

Snake venom phosphodiesterase is a single polypeptide with a molecular weight of 115,000–120,000 as judged by sodium do-

decyl sulfate-polyacrylamide gel electrophoresis (17,18). It requires 15 mM magnesium ions to show optimal activity, and has a broad pH-activity optimum (19). The enzyme is capable of hydrolyzing both DNA and RNA (20), removing the 5'-mononucleotide units from a polynucleotide chain in a stepwise fashion from the end that bears a free 3'-hydroxyl group (19). It is generally assayed by pH-stat titration where the quantity of phosphodiester bonds hydrolyzed is calculated from the amount of alkali consumed (21). The enzyme can also hydrolyze di-, tri-, and oligonucleotides as well. In some of these cases a spectrophotometric assay of the hydrolysis of *p*-nitrophenyl esters of oligonucleotides (22) or 4-methylumbelliferyl esters of mononucleotides (23) was possible.

The fluorescent changes that occur upon α - β phosphoryl bond cleavage of the 1,5-ANS-NTP nucleotide analogs provide for a sensitive, convenient, and rapid steady-state assay. The speed of the assay performed under stopped-flow conditions combined with computerized data storage and analysis allows both quantitative evaluation within seconds and if desired the continuous redesign of experimental strategy. While assays based upon release of nitrophenolate or 4-methylumbelliferone are of use over a narrow pH range, the sensitivity of this fluorescent assay

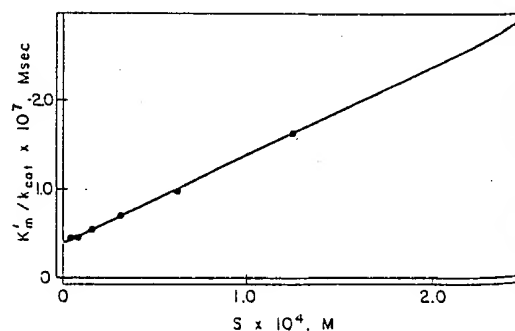


FIG. 6. Determination of the kinetic parameter K_p for TMP from the hydrolysis of 1,5-ANS-TTP by snake venom phosphodiesterase in 0.015 M MgCl_2 , 0.05 M Tris, pH 8.0, at 20°C . The ordinate, K'_m/k_{cat} , is obtained from the intercept of Eq. [1], where K'_m is $(1 + S/K_p)K_m$.

TABLE 3

KINETIC CONSTANTS FOR SNAKE VENOM PHOSPHODIESTERASE CATALYZED HYDROLYSIS OF 1,5-ANS-NTP

Nucleotide analog	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} s^{-1}$)	K_p (μM)
1,5-ANS-dCTP	630 (630)	18.8 (21.0)	33.5 (30.0)	46.7
1,5-ANS-dTTP	604 (555)	17.2 (19.2)	35.1 (28.9)	20.9
1,5-ANS-dTTP ^a	590 (500)	36.9 (30.3)	16.0 (16.5)	37.8
1,5-ANS-ATP	337	16.0	21.1	
1,5-ANS-CTP	198 (142)	10.9 (10.5)	18.2 (13.5)	12.0
1,5-ANS-GTP	609 (383)	9.2 (22.1)	66.2 (17.3)	25.4
1,5-ANS-UTP	213 (277)	4.2 (4.8)	50.7 (57.7)	6.1

Note. Assay conditions were 0.05 mM Tris, pH 8.0, 0.15 mM $MgCl_2$, 20°C. k_{cat} is based on an estimated purity of 10% for the commercial enzyme and 80% for the blue sepharose purified enzyme as determined by silver-stained sodium dodecyl sulfate-gels. The values in parentheses and values for K_p are calculated from progress curve analysis. All other kinetic constants are determined from initial rate analysis of the first 10% of the reactions.

^a Parameters are for blue Sepharose purified enzyme.

acrylamide gel electrophoresis requires 15 mM magnesium for optimal activity, and has a pH optimum (19). The enzyme is active in the presence of hydrolyzing both DNA and RNA by removing the 5'-mononucleotide from a polynucleotide chain in a reaction from the end that bears a phosphate group (19). It is generally used in a titration where the quantity of diester bonds hydrolyzed is proportional to the amount of alkali consumed. The enzyme can also hydrolyze oligonucleotides as well. It is used in a spectrophotometric assay for the hydrolysis of *p*-nitrophenyl esters (22) or 4-methylumbelliferyl mononucleotides (23) was

not affected over a wide pH range (Table 2). It may prove particularly useful for enzymes such as splenic phosphodiesterases which have a pH optimum of 5.6 to 5.9 (24). Initial rate measurements at 10% of reaction are used to determine K_m and k_{cat} for these analogs (Fig. 4). In addition, the integrated Michaelis-Menten equation (Eq. 1) can be solved to yield both the kinetic parameters and the value of K_p for nucleotide monophosphate (Figs. 5 and 6).

These fluorescent nucleotide analogs are excellent substrates for snake venom phosphodiesterase. They bind tightly with K_m 's ranging from 5 to 30 μM and are hydrolyzed rapidly (Table 3). The UTP analog binds about fivefold tighter than the best poly(U) substrate that has been made (25). Values of k_{cat} for these analogs are estimated to be in the range 200 to 600 s^{-1} based on a molecular weight of 120,000 for the protein.

As can be further seen from Table 3 the K_m for 1,5-ANS-TTP and the K_p for TMP differ only slightly from each other and from the value of 30 μM from the extrapolated data of Razzell and Khorana (22) or the value of 24 μM determined here by addition of TMP to the assay. Hence, it seems that the presence of the bulky naphthalene group does not significantly affect the ability of these

analogues to bind to snake venom phosphodiesterase. These results are in accord with the work of Khorana and co-workers (22) who concluded that modifications outside of the TMP moiety should have only slight effects on substrate binding. This further indicates that future changes in the type of fluorophore or distance from nucleotide moiety should not have a detrimental effect on the binding of the substrate to the enzyme.

This class of fluorescent nucleotides are also substrates for nucleotidyl transferring enzymes. The 1,5-ANS-ATP and 1,5-ANS-UTP derivatives have been shown to be good substrates for the *Escherichia coli* RNA polymerase, with only slightly increased K_m values and with V_{max} values of 50 to 70% that of normal nucleotides (26,27). In addition the ATP and UTP analogs are substrates for yeast RNA polymerase and the dGTP and TTP analogs are substrates for the reverse transcriptase from avian myeloblastosis virus (9).

While the fluorescent changes observed here upon α - β phosphoryl bond cleavage provides a convenient steady-state assay, it hinders their use for examination of ES complexes by RET. In order to optimally use the RET kinetic approach with an energy transfer relay system consisting of a fluorophore

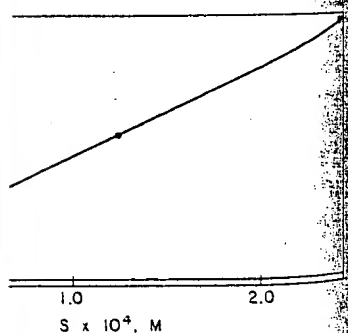


Figure 4. Determination of the kinetic parameter K_p for the hydrolysis of 1,5-ANS-TTP by snake venom phosphodiesterase in 0.015 M $MgCl_2$, 0.05 M Tris, 20°C. The ordinate, $K_p / (1 + S/K_p)$, is obtained from Eq. [1], where K_m is (1 + S/K_p) K_p .

in the substrate molecule and tryptophanyl residues of the enzyme, it is necessary that the fluorescence of the substrate and product fluorophore be the same (5). The purine nucleotide derivatives which exhibit relatively small fluorescence changes may still prove useful at enzyme concentrations of 1 to 10 μ M. Such enzyme concentrations will increase the RET signal, which is proportional to the concentration of the enzyme substrate complex, above the smaller fluorescence change observed as substrate is converted to product. However the very rapid turnover of the substrate will likely require low-temperature stopped-flow conditions for such mechanistic studies (12). We are presently purifying the enzyme to perform such experiments. In addition we are synthesizing nucleotides where there is a greater spacer arm between the fluorophore and the γ -phosphorus in the nucleotide in the hope of breaking the π conjugation between the nucleotide phosphates and the fluorophore. The use of these types of substrates for RET kinetics should further extend their usefulness in mechanistic studies of phosphodiesterases.

Snake venom phosphodiesterase shares a number of mechanistic features in common with the nucleotidyl transferases. All of these enzymes contain zinc, are activated by manganese and magnesium, and show a similar specificity toward the hydrolysis of an α - β phosphoryl bond. Mechanistic studies of phosphodiesterase may therefore be helpful in understanding the mechanistic details of the hydrolytic step involved in all of these enzymes.

ACKNOWLEDGMENTS

We are grateful to Dr. Bert L. Vallee for his continued advice and support and to Dr. T. C. French for writing the data analysis subroutines. The excellent technical assistance of Philip Maiorana is acknowledged.

REFERENCES

1. Auld, D. S. (1977) in *Biorganic Chemistry* (Van Tamelin, E. E., ed.), Vol. 1, pp. 1-17, Academic Press, New York.
2. Latt, S. A., Auld, D. S., and Vallee, B. L. (1972) *Anal. Biochem.* 50, 56-62.
3. Latt, S. A., Auld, D. S., and Vallee, B. L. (1972) *Biochemistry* 11, 3015-3022.
4. Lobb, R. R., and Auld, D. S. (1979) *Proc. Nat. Acad. Sci. USA* 76, 2684-2688.
5. Lobb, R. R., and Auld, D. S. (1980) *Biochemistry* 19, 5297-5302.
6. Auld, D. S. (1979) *Advances in Chemistry Series* Vol. 172, pp. 112-113.
7. Vallee, B. L. (1980) *Carlsberg Res. Commun.* 45, 423-441.
8. Hudson, E. N., and Weber, G. (1973) *Biochemistry* 12, 4154-4167.
9. Pollack, S. E., and Auld, D. S. (1980) *Fed. Proc.* 39, 2242.
10. Williams, E. J., Sung, S., and Laskowski, Sr. (1961) *J. Biol. Chem.* 236, 1130-1134.
11. Sulkowski, E., and Laskowski, M., Sr. (1971) *Biochim. Biophys. Acta* 240, 443-447.
12. Hanahan, D., and Auld, D. S. (1980) *Anal. Biochem.* 108, 86-95.
13. *Handbook of Biochemistry* (Saber, H. A., and Harte, R. A., eds.), p. G-53. Chem. Rubber Co., Cleveland.
14. Lineweaver, H., and Burk, D. (1934) *J. Amer. Chem. Soc.* 56, 658-666.
15. Jennings, R. R., and Nieman, C. (1955) *J. Amer. Chem. Soc.* 77, 5432-5433.
16. Woolf, B. (1932) cited by Haldane, J. B. S., and Stern, K. G. in *Allgemeine Chemie der Enzyme*, pp. 119-120, Steinkopff, Dresden/Leipzig.
17. Philipps, G. R. (1975) *Hoppe-Seyler's Z. Physiol. Chem.* 356, 1085-1096.
18. Oka, J., Ueda, K., and Hayaiski, O. (1978) *Biochem. Biophys. Res. Commun.* 80, 841-848.
19. Iwanaga, S., and Suzuki, T. (1979) in *Snake Venoms* (Lee, C., ed.), pp. 61-93, Springer-Verlag, Berlin/Heidelberg/New York.
20. Laskowski, M., Sr. (1971) in *The Enzymes* (Boyer, P. D., ed.), Vol. 4, pp. 313-328, Academic Press, New York.
21. Hadjiolov, A. A., Dolopchiev, L. B., and Milchev, G. I. (1966) *C. R. Acad. Bulg. Sci.* 19, 949-952.
22. Razzell, W. E., and Khorana, G. H. (1959) *J. Biol. Chem.* 234, 2105-2113.
23. Lo, K. W., Ferrar, W., Fineman, W., and Tsou, K. C. (1972) *Anal. Biochem.* 47, 609-613.
24. Bernardi, A., and Bernardi, G. (1971) in *The Enzymes* (Boyer, P. D., ed.), Vol. 4, pp. 329-336, Academic Press, New York.
25. Dolopchiev, L. B. (1969) *FEBS Lett.* 2, 185-188.
26. Yarbrough, L. R., Schlegeck, J. G., and Boughnam, M. (1979) *J. Biol. Chem.* 254, 12069-12073.
27. Yarbrough, L. R., and Block, J. L. (1980) *J. Biol. Chem.* 255, 9907-9911.
28. Knorre, D. G., Kurbatov, V. A., and Samukov, V. V. (1976) *FEBS Lett.* 70, 105-108.

NH₄HCO₃). The result was invariably a single fluorescent spot with the mobility of the original labeled peptide.

Our results thus provide a molecular weight limit for permeation of the junctional membrane channels. The permeating molecules were all short, simple, water-soluble peptide chains and hence were of extended form. Without further physical studies of the molecules themselves, it is not possible to determine from this weight limit a precise bore size of the channels. But the approximate bore size can be bracketed between the sizes of two limiting geometries of the largest permeant molecule, a sphere, representing the largest cross section, and a prolate spheroid with a major diameter of 30 Å, the upper limit of molecular extension. Thus, the effective channel diameter lies approximately between 14 and 10 Å (13). This is in satisfying agreement with a coarser estimate of the channel size based on electrical measurements. This estimate, based on the conductance of a minute junctional area (including the conductance component due to electrostatic interaction between channels) and the spacing of intramembranous particles of gap junction (widely assumed to contain the channels), gave a lower limit of conductance of 10⁻¹⁰ mho for the junctional channel unit and a lower limit of the channel diameter of the order of 10 Å (14).

IAN SIMPSON
BIRGIT ROSE

WERNER R. LOEWENSTEIN

Department of Physiology and
Biophysics, University of
Miami School of Medicine,
Miami, Florida 33152

References and Notes

- W. R. Loewenstein and Y. Kanno, *J. Cell Biol.* 22, 565 (1964).
- Y. Kanno and W. R. Loewenstein, *Nature (London)* 212, 629 (1966).
- D. D. Potter, E. J. Furshpan, E. S. Lennox, *Proc. Natl. Acad. Sci. U.S.A.* 55, 328 (1966); B. W. M. Payton, M. V. L. Bennett, G. D. Pappas, *Science* 166, 1641 (1969); R. Johnson and J. D. Shendan, *ibid.* 174, 717 (1971); for different techniques, see also J. Pitts [in *Ciba Symposium Growth Control in Cell Culture*, G. Wolstenholme and J. Knight, Eds. (Churchill & Livingston, London, 1971), p. 89; *Nature (London)* 255, 371 (1975)] and E. Rieske, P. Schubert, G. W. Kreutzberg [*Brain Res.* 84, 365 (1975)].
- For a definition of the junctional channel unit, see W. R. Loewenstein [*Ann. N.Y. Acad. Sci.* 137, 441 (1966)] and (14); for its morphological aspects, see N. B. Gilula [in *The Nervous System*, D. Tower, Ed. (Raven, New York, 1975), vol. 1], D. Goodenough [*Cold Spring Harbor Symp. Quant. Biol.* 40, 37 (1975)], C. Peracchia and M. E. Fernández-Jaimovich, *J. Cell Biol.* 67, 330a (1975).
- R. C. Nairn, *Fluorescent Protein Tracing* (Williams & Wilkins, Baltimore, 1969).
- The concentrations of the fluorescent compounds inside the micropipettes were 10⁻⁴M. The steady state concentrations inside the cells (upon dilution of the injected solutions into an average of ten cells) are estimated to range from 10⁻⁴M to 10⁻⁵M.
- B. Rose and W. R. Loewenstein, *J. Membr. Biol.* 28, 87 (1976).
- W. R. Loewenstein, M. Nakas, S. J. Socolar, *J. Gen. Physiol.* 50, 1865 (1967); G. M. Oliveira-Castro and W. R. Loewenstein, *J. Membr. Biol.* 5, 51 (1971).
- The pattern of spread of these molecules here in the normal, untreated cells was like that of the smaller molecules (≤ 1158 dalton) in cells whose cytoplasmic Ca²⁺ concentration was elevated—the tracers spread throughout the injected cell, but not beyond its junctional boundaries.
- On the other hand, the gland lumen, which is open to the exterior through the gland duct and has a depth comparable to the cells, became strongly fluorescent. Furthermore, dead cells became as fluorescent as the duct under these conditions. Dead cells were recognized in bright field by their swollen and granular appearance, and their enlarged nucleus and chromosomes.
- A small labeled fragment seemed unlikely because, as was already mentioned, the rates of intracellular movement of the tracer varied inversely as their presumed molecular weights.
- In this condition one would expect maximum lysosome breakage and, hence, maximum peptidase activity.

The size (2r) for the spherical molecular shape as determined from

$$r = \left(\frac{3 \text{ mol wt} \times \bar{v}}{4\pi N} \right)^{1/3}$$

where N is Avogadro's number and \bar{v} is the specific volume assumed to be 0.7; and the size for the most extended shape, was determined as the small diameter of the corresponding prolate spheroid whose major diameter was obtained with the aid of molecular models. The actual channel bore lies probably closer to the upper bracketing value (14 Å); for the molecules labeled with LBR and FITC, the small diameter of a realistic axiosymmetric equivalent is fixed at about this value by the labels themselves.

- W. R. Loewenstein, *Cold Spring Harbor Symp. Quant. Biol.* 40, 49 (1975).
- We thank Dr. L. Lorand for suggestions and for fibrinopeptide A and Dr. S. J. Socolar for discussion. This work was supported by research grant CA 14464 from the Public Health Service.

16 July 1976; revised 22 September 1976

Defined Dimensional Changes in Enzyme Cofactors:

Fluorescent "Stretched-Out" Analogs of Adenine Nucleotides

Abstract. A concept is presented for testing the dimensional restrictions of enzyme-active sites by stretching the substrate or cofactor by known magnitude. These restrictions of enzyme-active sites specific for purine cofactors were tested by the synthesis and evaluation of *lin*-benzoadenosine 5'-triphosphate, 5'-diphosphate, and 3',5'-monophosphate with respect to enzyme binding and activity. These "stretched-out" (by 2.4 angstroms) versions of the adenine ribonucleotides bind strongly, slow the enzymatic rates, and have useful fluorescence properties.

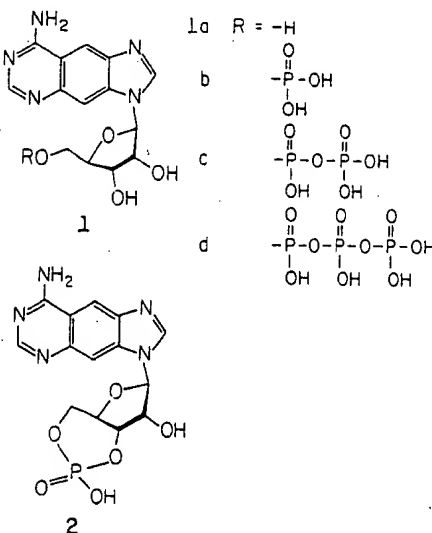
Laterally extended adenine nucleotides have been designed to examine the dimensional restrictions of enzyme-active sites specific for purine cofactors. One structural modification of this type involves the formal insertion of a benzene ring (actually four carbons) into the center of the purine ring system. In this way enzyme-binding characteristics at the terminal rings are preserved but are further separated by 2.4 Å while, at the same time, the potential for π interaction is increased. Initial experiments examining the substrate activity of *lin*-benzoadenosine (1a) (1, 2) and *lin*-ben-

zoadenine with a range of enzymes (3) demonstrated that such defined adjustments in the molecular periphery can help set limitations on the size and flexibility of the enzyme binding sites.

In view of these results it was anticipated that the enzymatic evaluation of the *lin*-benzoadenine nucleotides would also be informative since many enzymes utilize adenine nucleotides as substrates, cofactors, or allosteric effectors. In addition, it can be foreseen that the concept of defined dimensional changes is applicable to the construction and study of inhibitors. Furthermore, *lin*-benzoadenosine and its derivatives exhibit satisfactory fluorescence properties (a quantum yield of 0.44; a fluorescence lifetime of 3.7 nsec), and the nucleotides show sensitivity of the fluorophore to environmental conditions, such as divalent metal ions and stacking.

lin-Benzoadenosine (1a) was converted to its 5'-monophosphate derivative (1b) by reaction with pyrophosphoryl chloride according to the procedure described by Imai *et al.* (4). The integrity of the 5'-phosphorylation was established (i) by observing complete conversion of the *lin*-benzoadenosine 5'-monophosphate to the nucleoside (1a) on incubation with 5'-nucleotidase (5) and (ii) by ³¹P NMR (nuclear magnetic resonance) spectroscopy.

lin-Benzoadenosine diphosphate (1c)



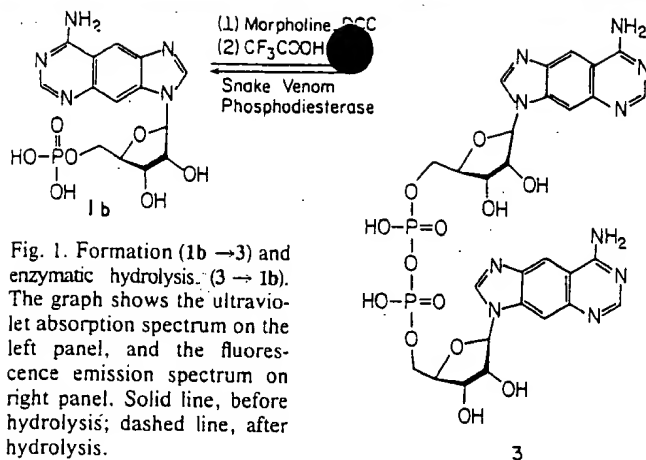
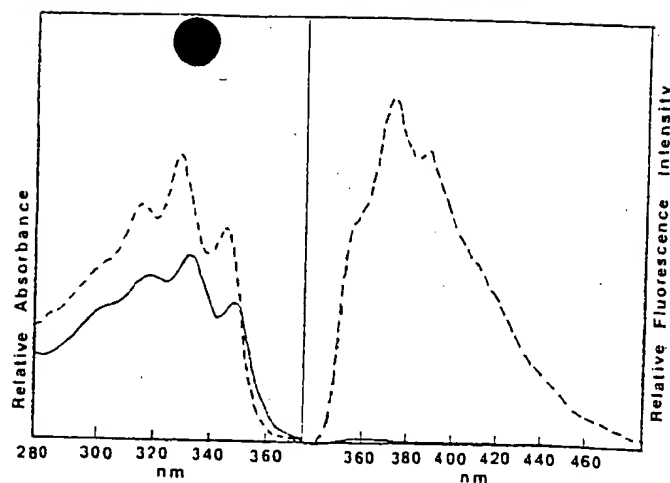


Fig. 1. Formation (1b → 3) and enzymatic hydrolysis (3 → 1b). The graph shows the ultraviolet absorption spectrum on the left panel, and the fluorescence emission spectrum on right panel. Solid line, before hydrolysis; dashed line, after hydrolysis.



and triphosphate (1d) were prepared from *lin*-benzoadenosine 5'-monophosphate by the phosphoromorpholidate method (6). Essentially quantitative conversion of the diphosphate to the triphosphate can be achieved enzymatically with pyruvate kinase. *lin*-Benzoadenosine 3',5'-monophosphate (2) was synthesized from *lin*-benzoadenosine via the trichloromethylphosphonate derivative (7). The identity and purity of the new *lin*-benzoadenosine nucleotides (1b to 1d and 2) were established by ³¹P NMR spectroscopy, high performance liquid chromatography, and electrophoresis. The structure of the 3',5'-monophosphate was further confirmed by its conversion to 1b on incubation with beef heart 3',5'-nucleotide phosphodiesterase (5), an enzyme that plays an important role in regulating intracellular cyclic adenosine monophosphate (cAMP). With this enzyme, the initial rate of hydrolysis of *lin*-benzoadenosine 3',5'-monophosphate (at 0.5 mM concentration) was approximately 5 percent of that for cyclic AMP.

To initiate our studies on the biological activity of the "stretched-out" nucleotides we have selected a representative group of kinases comprising pyruvate kinase, phosphofructokinase, phosphoglycerate kinase, and hexokinase. These enzymes, which exhibit broad to moderate nucleotide specificity, serve as representatives for measuring the degree to which *lin*-benzoadenosine 5'-diphosphate and *lin*-benzoadenosine 5'-triphosphate can function in enzyme systems. Conditions were selected to give consistent results for both adenosine triphosphate (ATP) and *lin*-benzoadenosine triphosphate and adenosine diphosphate (ADP) and *lin*-benzoadenosine diphosphate without seeking to achieve those of maximal activity for 1d or 1c (8).

We have found that *lin*-benzoadenosine diphosphate serves as sub-

strate for pyruvate kinase (rabbit muscle) with a K_m (Michaelis constant) of 0.74 mM compared to 0.30 mM for adenosine diphosphate (ADP) and a V_{max} equal to 20 percent of that for ADP. These data directed the use of the coupled assay for phosphofructokinase involving pyruvate kinase and lactate dehydrogenase. Proposed binding models (9) of pyruvate kinase for nucleotide substrates and fluorescence polarization studies with ϵ ADP (10) indicate that the base moiety of the substrate is not strongly associated with the protein. In view of this and the known broad specificity (11) of pyruvate kinase, it is not surprising that a lateral extension of the adenine nucleus is acceptable to this enzyme.

The next enzyme examined was phosphofructokinase (PFK, rabbit muscle) which requires ATP to phosphorylate fructose 6-phosphate. At low concentrations of *lin*-benzoadenosine triphosphate and ATP the K_m values for the cofactors were determined to be 0.16 mM and 0.04 mM, respectively, while the V_{max} values were of comparable magnitude. PFK is able to utilize several nucleoside triphosphates as phosphoryl donors (5). While these mainly comprise purine nucleotides, uridine triphosphate (UTP) and ϵ ATP (12) have also been shown to serve as cofactors. Acceptance of *lin*-benzoadenosine triphosphate by PFK represents the largest dimensional deviation known from the natural cofactor. At high concentrations of ATP (and UTP or ϵ ATP) PFK is significantly inhibited (13); *lin*-benzoadenosine triphosphate exhibits allosteric inhibition of this enzyme to approximately the same degree as ATP.

Yeast hexokinase, which exhibits more stringent nucleotide specificity, was assayed by the standard procedure of coupling to glucose-6-phosphate dehydrogenase. *lin*-Benzoadenosine 5'-triphosphate (1d) replaces ATP with this

enzyme; but, while the K_m values are of the same order (0.18 mM and 0.09 mM, respectively), the reaction rate with the "stretched-out" cofactor is approximately 40 times slower. Our kinetic data suggest that the "stretched-out" analog of ATP can be accommodated at the coenzyme binding site, but that there is a reduction in the efficiency of phosphoryl transfer to the substrate, possibly because of greater steric restrictions within the glucose and coenzyme binding regions. Hexokinase has an intrinsic hydrolytic activity that is low compared with the glucose phosphorylating activity (14). The occurrence of adenosine triphosphatase (ATPase) activity makes it essential to carry out binding measurements rapidly, a problem that has yet to be satisfactorily solved. *lin*-Benzoadenosine triphosphate may provide a means of studying the interaction of cofactor and hexokinase since it can be accommodated at the coenzyme binding site, while its slow reactivity makes it eminently suitable for fluorescence polarization measurements.

Finally, we examined the ability of *lin*-benzoadenosine triphosphate to phosphorylate 3-phosphoglyceric acid, catalyzed by yeast 3-phosphoglycerate kinase (PGK), in comparison with ATP. The phosphorylation was assayed according to the standard procedure of coupling the reaction to glyceraldehyde-3-phosphate dehydrogenase. *lin*-Benzoadenosine triphosphate functioned in the system with a K_m of 0.4 mM, while under identical conditions the K_m observed for ATP was 0.5 mM. The V_{max} value for the "stretched-out" analog was approximately 1 percent of that of ATP. This activity of the *lin*-benzoadenosine triphosphate in the PGK system has permitted the enzymatic synthesis of *lin*-benzoadenosine 5'-[γ -³²P]triphosphate (15), the availability of which facilitates further enzymatic studies.

A considerable body of evidence has now been presented that the nucleotide-binding sites of PGK and several dehydrogenases (lactate, malate, alcohol, and glyceraldehyde-3-phosphate dehydrogenases) are alike (16) and it is thus attractive to speculate from our data that the *lin*-benzo analog of NAD⁺ (nicotinamide adenine dinucleotide) will bind to these dehydrogenases.

The lateral extension of the purine ring system has provided a heterocycle with interesting spectroscopic properties. For example, evidence for an intramolecular interaction of the 5'-phosphate substituent with the chromophore comes from the spectroscopically determined pK_a values (base protonation) for the "stretched-out" analogs of adenine nucleotides in aqueous solution: *lin*-benzoadenosine triphosphate, pK_a , 7.1 (6.6 in presence of 5 mM Mg²⁺); *lin*-benzoadenosine diphosphate, pK_a , 7.3 (6.9 in presence of 5 mM Mg²⁺); *lin*-benzoadenosine monophosphate, pK_a , 7.6 (unchanged in 5 mM Mg²⁺); *lin*-benzoadenosine 3',5'-phosphate, pK_a , 5.6 (unchanged in 5 mM Mg²⁺); and *lin*-benzoadenosine, pK_a , 5.6 (unchanged in 5 mM Mg²⁺). When intramolecular interaction of the phosphate and the base is not possible, as in *lin*-benzoadenosine 3',5'-monophosphate, no change in pK_a is observed with respect to that of *lin*-benzoadenosine. These data suggest participation of the phosphates in base protonation. The lowering of the pK_a values in the presence of 5 mM Mg²⁺ indicates the formation of magnesium chelate complexes with the polyphosphate residues of *lin*-benzoadenosine di- and triphosphates. At the pH at which all our enzyme studies have been conducted (pH 7.5, 5 mM Mg²⁺) the "stretched-out" base moiety of the di- and triphosphates can be considered mainly in the unprotonated form.

In acidic aqueous solutions of *lin*-benzoadenosine triphosphate, the fluorescence emission shifted to longer wavelength—385 nm compared to 372 nm (corrected values) for the unprotonated form, pK_a^* being close to the ground state pK_a —with little change in quantum yield. This makes the usefulness of the compound equally satisfactory over a wide pH range. The second protonation of the system takes place at approximately pH 1. The presence of the long wavelength absorption band permits excitation of the fluorophore without interference from any other ultraviolet-absorbing species in proteins and nucleic acids. The fluorescence emission spectra are also sensitive to the presence

of divalent cations. The addition of 5 mM Mg²⁺ alters the pK_a^* of *lin*-benzoadenosine triphosphate, as judged by the fluorescence emission spectra, without incurring significant change in fluorescence intensity. Fluorescence quenching by Co²⁺ in the case of *lin*-benzoadenosine di- and triphosphates is also indicative of divalent metal ion complexes with the polyphosphate residues and their interaction with the fluorophore.

We have been interested in intramolecular base-stacking interactions of the heteroaromatic bases of nucleic acids (17). With a variety of *lin*-benzoadenosine derivatives on hand, we therefore investigated the magnitude of the interaction between two of the tricyclic ring systems. The percentages of hypochromism for *P*¹,*P*²-di-*lin*-benzoadenosine-5'-pyrophosphate (3) and *P*¹,*P*²-diadenosine-5'-pyrophosphate were compared by means of hydrolytic cleavage with snake venom phosphodiesterase and were found to be 23 and 9 percent, respectively, at pH 8.5 and 25°C. The hydrolytic cleavage of *P*¹,*P*²-di-*lin*-benzoadenosine-5'-pyrophosphate also results in an increase in fluorescence intensity of approximately two orders of magnitude (Fig. 1). This dramatic change in fluorescence yield indicates that the *lin*-benzoadenosine moieties, when connected intramolecularly, can form dark complexes and can undergo intramolecular collisional quenching. It is therefore predictable that intramolecular positioning of a *lin*-benzoadenosine system in close proximity to other moieties—for example, nicotinamide and isoalloxazine—will result in fluorescence quenching.

Pilot studies indicate that *lin*-benzoadenosine 5'-diphosphate acts as a substrate for primer-independent polynucleotide phosphorylase (*Micrococcus luteus*) in the presence of Mn²⁺. The polymeric material isolated by gel chromatography was essentially nonfluorescent, and its long-wavelength band in the ultraviolet spectrum lacked the characteristic fine structure of the monomeric species (Fig. 1) and was broadened. The fluorescence and the fine structure of the long-wavelength absorption band returned on treatment with 0.1M KOH at 100°C or as a result of enzymatic cleavage (18), showing again the strong stacking interaction between tricyclic base units. It should now be possible to study polynucleotide binding for the purpose of testing new complementarity relationships.

For the enzymes investigated, the formal insertion of a benzene ring in the cen-

ter of the adenine nucleotides does not greatly diminish their binding properties with respect to those of the normal nucleotides, but usually decreases the rate of reaction. Overall, the series 1a to 1d and 2 exhibit significant biological activity, varying with different enzymes. In addition, the useful fluorescence properties of *lin*-benzoadenosine nucleotides and their increased π interactions can be directed to many studies of static and dynamic interactions with different moieties, complexation, the nature of enzyme binding sites, and conformational changes induced by surrogate coenzyme-enzyme binding.

DAVID I. C. SCOPES

JORGE R. BARRIO

NELSON J. LEONARD

School of Chemical Sciences,
University of Illinois,
Urbana 61801

References and Notes

1. N. J. Leonard, M. A. Sprecker, A. G. Morrice, *J. Am. Chem. Soc.* **98**, 3987 (1976).
2. The prefix *lin* refers to the linear disposition of the three rings [see (1)]; "benzo" in the trivial name refers to the additional ring which, only when central, contains no nitrogen. The other parts of the names follow accepted IUPAC-IUB nomenclature.
3. Selected enzymes included those that act on free bases and on the heteroaromatic portion of ribonucleosides as well as enzymes that are involved in ribosyl and phosphoribosyl transfer.
4. K. Imai, S. Fujii, K. Takanohashi, Y. Funikawa, T. Masuda, M. Honjo, *J. Org. Chem.* **34**, 1547 (1969).
5. P. D. Boyer, Ed., *The Enzymes* (Academic Press, New York, ed. 3, 1974), vol. 8.
6. J. G. Moffatt and H. G. Khorana, *J. Am. Chem. Soc.* **83**, 649 (1961).
7. R. Marimoto, T. Nishimura, M. Honjo, *Chem. Pharm. Bull. (Tokyo)* **23**, 2295 (1975).
8. The enzyme assays were monitored by ultraviolet absorbance at 370 nm rather than at the usual 340 nm, to minimize the background due to absorbance by the "stretched-out" base (1).
9. A. S. Mildvan and M. Cohn, *J. Biol. Chem.* **241**, 1178 (1966).
10. J. R. Barrio, J. A. Secrist, Y. Chien, P. J. Taylor, J. L. Robinson, N. J. Leonard, *FEBS Lett.* **29**, 215 (1973).
11. K. M. Plowman and A. R. Krall, *Biochemistry* **4**, 2809 (1965).
12. J. A. Secrist, J. R. Barrio, N. J. Leonard, *Science* **175**, 646 (1972); ———, G. Weber, *Biochemistry* **11**, 3499 (1972).
13. H. A. Lardy and R. E. Parks, in *Enzymes: Units of Biological Structure and Function*, O. H. Gaebler, Ed. (Academic Press, New York, 1956); K. Uyeda and E. Racker, *J. Biol. Chem.* **240**, 4682 (1965); J. A. Secrist, J. R. Barrio, N. J. Leonard, G. Weber, *Biochemistry* **11**, 3499 (1972); P. M. Lad, D. E. Hill, G. G. Hammes, *ibid.* **12**, 4303 (1973).
14. G. de la Fuente and A. Sols, *Eur. J. Biochem.* **16**, 234 (1970).
15. I. M. Glynn and J. B. Chappell, *Biochem. J.* **90**, 147 (1964).
16. I. Öhlsön, B. Nordström, C.-I. Brändén, *J. Mol. Biol.* **89**, 339 (1974); C. F. Blake and P. R. Evans, *ibid.* **84**, 585 (1974); M. Buehner, G. C. Ford, D. Moras, K. W. Olsen, M. G. Rossmann, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 3052 (1973).
17. N. J. Leonard and K. Ito, *J. Am. Chem. Soc.* **95**, 4010 (1973), and references cited therein.
18. An enzyme mix comprising *Micrococcus* nuclease, snake venom phosphodiesterase, and alkaline phosphatase.
19. This work was supported by grant GM-05829 from the National Institutes of Health. We thank Dr. M. A. Sprecker for initial preparation of 1b.

28 June 1976; revised 8 September 1976

BBA 46 105

SYNTHESIS OF 2-(DANSYLAMINO)ETHYL TRIPHOSPHATE AND ITS PROPERTIES AS A FLUORESCENT SUBSTRATE OF HEAVY MEROMYOSIN-ATPase

MASAHIKO ONODERA* AND KOICHI YAGI

Department of Chemistry, Faculty of Science, Hokkaido University, Sapporo (Japan)
(Received April 9th, 1971)

SUMMARY

2-(Dansylamino)ethanol, which was obtained after the reaction of dansyl chloride with ethanolamine, was phosphorylated by using tetrachloropyrophosphate, and the resulting 2-(dansylamino)ethyl monophosphate was further phosphorylated by using P_1 and N,N' -dicyclohexylcarbodiimide to obtain 2-(dansylamino)ethyl triphosphate (DTP).

DTP was hydrolyzed by heavy meromyosin-ATPase at a rate similar to the hydrolysis of ATP in the presence of Ca^{2+} . In the presence of 10 mM Mg^{2+} , the K_m of the DTP hydrolysis by heavy meromyosin was $1.9 \cdot 10^{-5}$ M, the hydrolysis apparently being abolished by the addition of ATP.

Marked increases in the intensity of the maxima of the excitation and emission spectra of DTP were observed after the addition of heavy meromyosin in the presence of 83.4 mM Mg^{2+} at 10° . The peak of the emission spectrum shifted from 540 to 530 nm accompanied by an increase in intensity, thus suggesting that the polarity around DTP became rather hydrophobic. Energy transfer from tryptophan and/or tyrosine to the dansyl group of DTP was assumed.

INTRODUCTION

The structure of the active site of myosin-ATPase (EC 3.6.1.3) has been investigated by using substrate analogs, all of which contained purine or pyrimidine bases or their derivatives. TONOMURA *et al.*¹ concluded from their study that the N-6 or O-6 of purine and pyrimidine bases is necessary for the interaction of the substrate analogs with the active site, and that the appropriate distance between the triphosphate and the base should be maintained for a desirable fit of the substrate analog with myosin.

On the other hand, it has been known since the observations of SINGER AND BARRON² and of KIELLY AND BRADLEY³ that the sulphydryl groups of myosin are

Abbreviations: DTP, 2-(dansylamino)ethyl triphosphate; DDP, 2-(dansylamino)ethyl di-phosphate; DMP, 2-(dansylamino)ethyl monophosphate.

* Present address: Biochemistry Division, Research Institute for Tuberculosis, Hokkaido University, Sapporo, Japan.

DTP: A FLUORESCENT SUBSTRATE OF MYOSIN-ATPase

255

essential for its ATPase activity. Recently, MURPHY AND MORALES⁴ have shown by affinity labelling to the -SH group using 6-mercapto-9- β -D-ribofuranosylpurine 5'-triphosphate as the substrate analog that low-molecular-weight components⁵ included in the myosin molecule may be indispensable for its enzymic activity.

As an approach to clarify the structure of the enzyme's active site, fluorescent substrate analogs have sometimes been used with success^{6,7}. It is now expected that fluorescent reagents will be most useful in the study of the enzyme's active site.

In this paper the synthesis of a new fluorescent substrate, 2-(dansylamino)ethyl triphosphate (DTP), of myosin-ATPase is presented. It was hydrolyzed by heavy meromyosin-ATPase at a comparable rate to the hydrolysis of ATP. The qualitative nature of DTP hydrolysis resembles more that of ITP than that of ATP. It was also shown that the environment around the bound DTP was rather hydrophobic. Some of the results have been presented elsewhere^{8,9}.

MATERIALS AND METHODS

Heavy meromyosin was prepared from rabbit skeletal muscle by the method similar to that described by SZENT-GYÖRGYI¹⁰.

The dephosphorylation reaction was stopped by addition of trichloroacetic acid at a final concentration of 5%, and the P_i liberated was determined by the methods of FISKE AND SUBBAROW¹¹ or MARTIN AND DOTY¹². The enzymatic activity of heavy meromyosin-ATPase measured at 25° was expressed in units, which were defined as μ moles P_i liberated per min per mg of protein. The concentration of heavy meromyosin was determined by using the absorbance at 280 nm of 0.63 for 1 mg protein per ml solution.

Paper electrophoresis was performed at pH 7-8 (50 mM triethylammonium bicarbonate buffer) and at 30 V/cm for 1-2 h using the Mitsumi electrophoresis instrument. Paper chromatography was performed by using Toyo Roshi 3 A paper (16 cm \times 60 cm), following the descending technique. The spots were detected under ultraviolet light from a Manasul lamp.

The carbonate form of DEAE-cellulose was soaked in 0.5 M ammonium carbonate for 1-2 h with stirring and then washed with water in a column (2 cm \times 60 cm) until the wash water became neutral. The height of DEAE-cellulose (carbonate form) in the column was 47 cm.

The P_i content in 2-(dansylamino)ethyl phosphates was analyzed by the method of ALLEN¹³.

Absorption spectra were measured with a Hitachi 124 spectrophotometer. Fluorescence measurements were carried out with a Hitachi 2PF-3A spectrofluorimeter and the temperature of the cell holder was regulated by circulating water.

The fluorescence spectra shown in this paper were not corrected for the spectral response of the photomultiplier and the Xenon lamp. Excitation bandwidths were 3 or 4 nm and the emission band width was 12 nm.

NMR spectra were measured by a JEOL 3H 60 high resolution NMR spectrometer.

Organic solvents were all purified by distillation.

ATP was purchased from Sigma Chemical Company. 1-Dimethylaminonaphthalene-5-sulfonyl chloride (dansyl chloride) was purchased from Mann Research Laboratories.

RESULTS

Preparation of 2-(dansylamino)ethanol

3.5 g of ethanolamine were added to an equal amount of dansyl chloride dissolved in 300 ml of ethanol, and the mixture was allowed to stand for 2 h at room temperature. The solution was evaporated to 50 ml by heating it to 80–85°. After cooling, a small amount of water was added to the solution and a precipitate of the product appeared. By further addition of water to 2 l, the precipitate was again solubilized and a solution giving green fluorescence was obtained. The solution was filtered through a glass filter, and the filtrate was evaporated to 300 ml at 100°. Yellowish green crystals were obtained from the solution after standing overnight in the cold room. The precipitate was collected and dried in a desiccator. Yield was 94%. The molar absorption coefficient of the product at 320 nm was $5410 \text{ mole}^{-1} \cdot \text{cm}^{-1}$ (reference¹⁴ value: $5310 \text{ mole}^{-1} \cdot \text{cm}^{-1}$). Only one spot, whose R_F was 0.9, was obtained by paper chromatography developed by ammonium acetate-ethanol (2:7, by vol.). Melting point 100° (reference¹³: 102°). An NMR spectrum measured in dimethyl sulfoxide has shown alcoholic OH at the 4.65 ppm triplet, amide NH at the 7.9 ppm singlet, and naphthalene 6 protons at 7.1–8.6 ppm. The OH and NH signals disappeared by the addition of D_2O .

Preparation of 2-(dansylamino)ethyl monophosphate (DMP)

1 mmole of 2-(dansylamino)ethanol was dissolved in 22 ml of acetonitrile at -5° , 10 mmoles of tetrachloropyrophosphate were added slowly to the solution with stirring. The synthesis of DMP was followed by paper electrophoresis. After reaction for 2 h 2-(dansylamino)ethanol disappeared completely and one large spot moving to the anode was obtained, with small spots containing more anionic compounds. The reaction was stopped by adding 10 vol. of water. Derivatives of 2-(dansylamino)ethanol in the solution were adsorbed on to 2–3 g of charcoal by shaking vigorously. The charcoal was washed 3 times with 100 ml of a mixture of water-ethanol-benzene (4:8:1, by vol.), with vigorous stirring. 72% of the material could be desorbed. Benzene and ethanol were removed by evaporation under reduced pressure at 30°. The 2-(dansylamino)ethanol and its derivatives were fractionated by DEAE-cellulose column chromatography under a concentration gradient of triethylammonium bicarbonate buffer (pH 7.0). DMP was obtained as the first main peak at 0.14–0.20 M triethylammonium bicarbonate. The concentration of triethylammonium bicarbonate at the peak was rather variable due to evolution of CO_2 gas during the experiment. The yield from 2-(dansylamino)ethanol was 65%. Fractions of the first peak were collected and the solvent (triethylammonium bicarbonate) was removed by evaporation under reduced pressure at 30°. The residue was dissolved in a small volume of water. The dissolution-evaporation cycle was repeated until the pH of the aqueous solution became neutral. The final product showed only one spot on paper electrophoresis and also on paper chromatography (R_F , 0.65) developed by ammonium acetate-ethanol (2:7, by vol.). The molar ratio of dansyl group to P_1 in the DMP preparation was 1.0.

Preparation of DTP

Synthesis of DTP was performed according to the well-known condensation method of nucleotide monophosphate and P_1 using N,N' -dicyclohexylcarbodiimide

DTP: A FLUORESCENT SUBSTRATE OF MYOSIN-ATPase

as a condensing agent¹⁵. DMP and P_1 in final concentrations of 0.1 mM and 1 mM, respectively, were dissolved in 2 ml pyridine containing 2.1 mM tri-*n*-butylamine. Tri-*n*-butylamine was added to dissolve the P_1 in the pyridine. The DMP was converted from a triethylammonium salt to a pyridine salt by repeating the dissolution in pyridine-evaporation cycle. Phosphorylation of DMP was started by the addition of 50 mmoles of N,N' -dicyclohexylcarbodiimide with stirring. After continuous stirring in the dark at room temperature for 2 days, the light-yellow solution became white and turbid, the turbidity being due to the formation of cyclohexylurea. The precipitate was removed by filtration through a glass filter and was washed with an appropriate amount of water (20–30 ml). An equal volume of ethyl ether was added to the combined filtrate and the water layer was obtained after shaking 2 or 3 times. Any organic solvent in the water layer was removed by evaporation and the aqueous solution was fractionated by DEAE-cellulose column chromatography. As shown in Fig. 1, DTP was separated from DMP and 2-(dansylamino)ethyl diphosphate (P_2). Properties of the components of the first three peaks have not yet been examined. It is presumed that the components are 2-(dansylamino)ethanol and the pyro-type of DMP.

The yield of DTP from DMP was 78%. When the amount of N,N' -dicyclohexylcarbodiimide used was less than 50 mmoles, the yield of DTP was less. Only one spot was detected with the DTP preparation by paper electrophoresis and by paper chromatography. The molar ratio of the dansyl group to P_1 was 1:3.3. DTP was used as the triethylammonium salt. Aqueous DTP solution was rather stable, i.e. after 3 months in the deep freezer at -20° , more than 97% of the DTP remained undiluted.

Absorption and fluorescence spectra

Fig. 2 shows the absorption spectrum of 2-(dansylamino)ethanol. Absorption maxima were obtained at 215, 245, and 328 nm in 20 mM Tris-HCl buffer (pH 8.0).

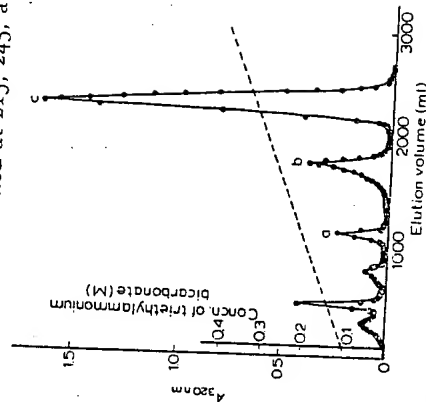


Fig. 1. DEAE-cellulose column chromatography of 2-(dansylamino)ethyl phosphates. Aqueous solution of a mixture of 2-(dansylamino)ethyl phosphates was applied to the column (COOH form, 2 cm X 47 cm). Elution was performed under a linear concentration gradient of triethylammonium bicarbonate from 0.1 to 0.4 M (-----). DMP(a), DDP(b), and DTP(c) were eluted at 0.20, 0.27, and 0.32 M triethylammonium bicarbonate, respectively. Fractions of 20 ml were collected. It took 36 h to complete this chromatographic fractionation. Three peaks eluted in front of DMP were not examined; see text.

The absorption spectra of phosphorylated derivatives of 2-(dansylamino)ethanol were indistinguishable from that of 2-(dansylamino)ethanol in the range 230 to 400 nm. Since the molar absorption coefficients of the phosphorylated derivatives at 328 nm were apparently identical with that of 2-(dansylamino)ethanol, absorbance at 328 nm of these compounds was adjusted to 0.03 and the fluorescence intensities, which were excited at 328 nm and measured at 540 nm, were compared. The fluorescence intensities of DMP, DDP and DTP were only 4, 2 and 1 % higher, respectively, than that of 2-(dansylamino)ethanol.

Increase in the concentration of ethanol up to 70 % at neutral pH increased 9-fold the intensity of the fluorescence of 2-(dansylamino)ethanol, which was measured in the range 440 to 620 nm (Fig. 3). At the same time, the peak of the fluorescence emission spectrum shifted from 540 to 523 nm. The fluorescence intensity of 2-(dansylamino)ethanol, which was excited at 328 nm and measured at 485 nm, was increased by the addition of bovine serum albumin in 40 mM Tris buffer (pH 7.8) at 25°. As shown in Fig. 4, the intensity at 485 nm increased 82-fold by the addition of the protein in a final concentration of 5 mg/ml. The fluorescence emission spectrum maximum was at 485 nm in the presence of 5 mg/ml of the protein, but it shifted to a longer wavelength with a decrease in protein concentration being at 540 nm in the absence of the protein.

A small decrease in the fluorescence intensity was observed by adding KCl or $MgCl_2$ to 2-(dansylamino)ethanol and its phosphorylated derivatives. When $MgCl_2$ was added in a final concentration of 0.2, 0.5 or 1.0 M, the extent of quenching of phosphorylated derivatives was 9, 20 or 28 %, respectively, but when the same

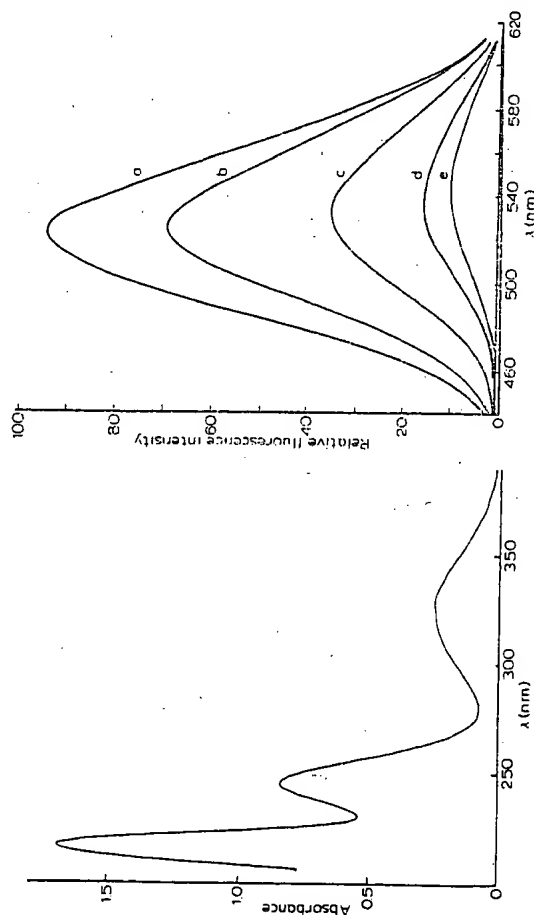


Fig. 2. Absorption spectrum of 2-(dansylamino)ethanol. Concentration of 2-(dansylamino)ethanol was $5.2 \cdot 10^{-5}$ M. Solvent: 20 mM Tris-HCl buffer (pH 8.0).

Fig. 3. Effect of ethanol on the fluorescence emission spectrum of 2-(dansylamino)ethanol. Excitation at 328 nm. Concentration of ethanol in percentage (v/v): a, 70; b, 50; c, 30; d, 10; e, none. Concentration of 2-(dansylamino)ethanol was $6.4 \cdot 10^{-6}$ M.

concentrations were added to 2-(dansylamino)ethanol itself, quenching was 4, 13 or 22 %, respectively. No difference in the effect of KCl was observed between 2-(dansylamino)ethanol and the phosphorylated derivatives, and the quenching with 2 M KCl was less than 15 %. In the presence of 0.5 M KCl and 100 mM $MgCl_2$, the quenching of DTP was 5-10 %.

Fig. 5 shows the effect of pH on the fluorescence intensity of DTP, which was excited at 328 nm and measured at 540 nm. The quenching of dansyl group fluorescence by decreasing the pH to the acid region has already been reported by FÖRSTER¹⁶. It was explained by the protonation of the dimethylamino group. Quenching of the fluorescence intensity of DTP was clearly observed below pH 6, while no fluorescence was observed below pH 3. The absorption at 328 nm was also decreased by decreasing the pH.

Hydrolysis of DTP by heavy meromyosin

Hydrolysis of DTP by heavy meromyosin was measured in a reaction mixture containing 0.1 mg/ml heavy meromyosin, 0.3 mM DTP, 4.85 mM $CaCl_2$, 0.5 M KCl and 50 mM Tris-maleate buffer (pH 7.0), at 25°. As shown in Fig. 6, P_i was liberated from DTP at the same rate as from ATP. P_i liberation from DDP under the same conditions was negligible. P_i liberation from DTP was hardly observed when heavy meromyosin which was inactivated by preincubation in 0.5 M KCl (pH 7.0) at 80°

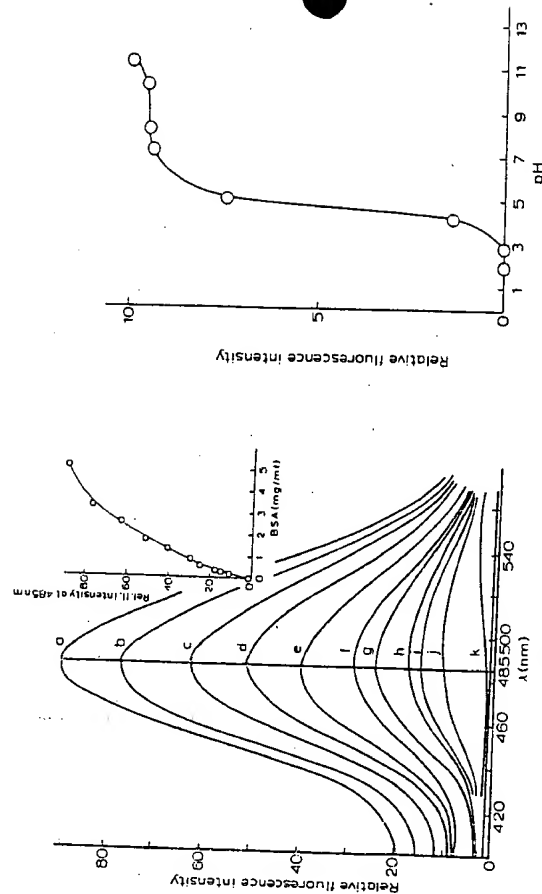


Fig. 4. Effect of bovine serum albumin on fluorescence emission spectrum of 2-(dansylamino)ethanol. Concentration of 2-(dansylamino)ethanol was $1.7 \cdot 10^{-6}$ M. Concentration of bovine serum albumin in mg/ml: a, 5.0; b, 3.3; c, 2.5; d, 1.7; e, 1.25; f, 0.84; g, 0.63; h, 0.42; i, 0.31; j, 0.21; k, none. Inset shows the relation between the fluorescence intensity at 485 nm and the concentration of bovine serum albumin (BSA).

Fig. 5. Effect of pH on the fluorescence intensity of DTP. The pH's above 10 were adjusted by KOH and pH 2 was adjusted by HCl. 20 mM citrate buffer was used for the pH in the range from 2.8 to 6.0, and 20 mM Tris-HCl buffer was used from pH 7.1 to 8.1. The absorbance of DTP ($6.4 \cdot 10^{-6}$ M) at 328 nm was 0.03 in the solution at pH 7.6.

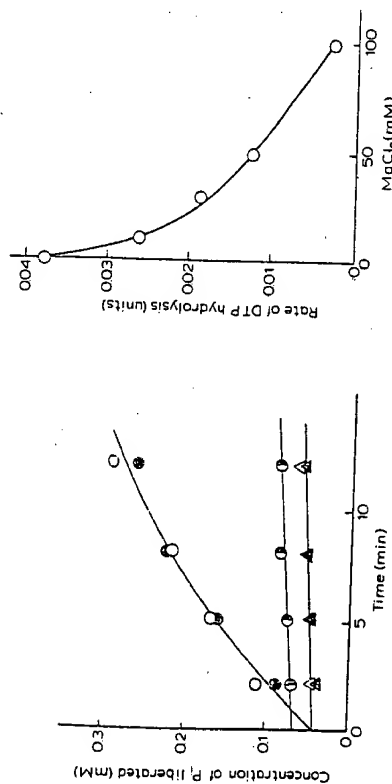


Fig. 6. DTP hydrolysis by heavy meromyosin. Reaction mixture consisted of 0.1 mg/ml heavy meromyosin, 0.3 mM DTP (or ATP, DDP), 0.5 M KCl, 4.85 mM CaCl_2 , and 50 mM Tris-maleate buffer (pH 7.0). Temperature, 25°. \bullet , Δ : ATP; \circ , Δ : DDP. \blacktriangle , \triangle : heat-denatured heavy meromyosin was used.

Fig. 7. Effect of Mg^{2+} inhibition on DTP hydrolysis by heavy meromyosin. Reaction mixture consisted of 0.1 mg/ml heavy meromyosin, 0.3 mM DTP, 0.5 M KCl, 20 mM Tris-HCl buffer (pH 7.6) and various concentrations of MgCl_2 . Temperature, 9.8°.

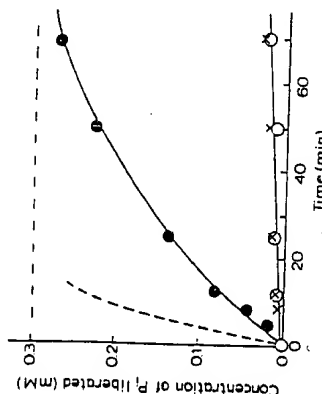


Fig. 8. Inhibitory effect of ATP on DTP hydrolysis by heavy meromyosin. Reaction mixture consisted of 0.02 mg/ml heavy meromyosin, 0.3 mM DTP and/or ATP, 0.5 M KCl, 10 mM MgCl_2 , and 50 mM Tris-maleate buffer (pH 7.0). Temperature, 25°. Substrate: \bullet , DTP; \circ , ATP; \times , 0.3 mM DTP and 0.3 mM ATP. Broken line, time course of P_i liberation from ATP in the presence of Ca^{2+} in place of Mg^{2+} . The vertical broken line shows the level of DTP concentration added.

Fig. 9. Effect of NaCl or KCl concentration on DTP hydrolysis by heavy meromyosin. Reaction mixture used for the experiment with NaCl consisted of 0.05 mg/ml heavy meromyosin, 0.58 mM DTP, 10 mM MgCl_2 , 20 mM Tris-HCl buffer (pH 7.6) and various concentrations of NaCl (\bullet). Temperature, 9°. Reaction mixture used for the experiment with KCl consisted of 0.025 mg/ml heavy meromyosin, 0.93 mM DTP, 4.85 mM CaCl_2 , 20 mM Tris-HCl buffer (pH 7.6) and various concentrations of KCl (\circ). Temperature, 25°. In order to save the DTP its concentration was kept as low as possible. Reaction mixture used for ATP hydrolysis in the presence of KCl consisted of 0.025 mg/ml heavy meromyosin, 2 mM ATP, 5 mM CaCl_2 , 50 mM Tris-maleate buffer (pH 7.0) and various concentrations of KCl (\circ). Temperature, 25°.

DTP: A FLUORESCENT SUBSTRATE OF MYOSIN-ATPase

261

for 60 min was used in place of native heavy meromyosin. As shown in Fig. 7, DTP hydrolysis was inhibited by the addition of MgCl_2 in 0.5 M KCl and 20 mM Tris-HCl buffer (pH 7.6) at 9.8°. 70% of the original activity was preserved after the addition of MgCl_2 in a final concentration of 10 mM, but only 7% or less was observed in the presence of 100 mM MgCl_2 . The rate of ATP hydrolysis by heavy meromyosin in 0.35 M KCl at pH 8.2 decreased to less than 30% of the original activity with the addition of MgCl_2 in a final concentration of 10 mM (ref. 17). As shown in Fig. 8, ATP hydrolysis was inhibited nearly completely in the presence of 10 mM MgCl_2 , while under the same conditions DTP hydrolysis was clearly shown. When both DTP and ATP were added at an equal concentration of 0.3 mM, the rate of P_i liberation was the same as that obtained in the presence of ATP as the only substrate. The result strongly suggests that ATP and DTP compete for the same active site and that the affinity of ATP for the site is stronger than that of DTP. This conclusion was confirmed by the measurement of K_m 's for ATP and DTP as described below.

Heavy meromyosin-ATPase activity in the presence of Ca^{2+} is simply decreased by increasing the concentration of KCl. When DTP was used as the substrate in place of ATP, the level of P_i liberation was nearly one-half that of ATP hydrolysis in the absence of KCl. As shown in Fig. 9, this level increased with increase in the concentration of KCl and, after the maximum activity was attained at 0.2 M KCl, it gradually decreased. Graphs of the decrease in the rate of ATP hydrolysis and of the increase in DTP hydrolysis intersected at 0.1 M KCl. The rate of DTP hydrolysis was also examined in the presence of 10 mM MgCl_2 at various concentrations of NaCl (Fig. 9). An effect similar to that of KCl was obtained and the maximum activity was attained at 0.3–0.4 M NaCl.

DTP was not hydrolyzed by heavy meromyosin in the presence of 10 mM EDTA and 0.5 M KCl at pH 7, 25°, while ATP hydrolysis was activated under the same conditions.

The Michaelis constant (K_m) and the maximum velocity (v_{\max}) of DTP hydrolysis by heavy meromyosin were measured in the presence of Mg^{2+} or Ca^{2+} at pH 7.0 and 25°. In the presence of 10 mM MgCl_2 and 0.5 M KCl, the K_m was $1.9 \cdot 10^{-5}$ M and v_{\max} was 0.24 unit. In the presence of 4.85 mM CaCl_2 and 0.5 M KCl, the K_m was found to be $1.8 \cdot 10^{-4}$ M and v_{\max} was 1.56 units. In the case of ATP hydrolysis by heavy meromyosin, K_m and v_{\max} with 2 mM Mg^{2+} were less than 10^{-6} M and 0.1 unit, respectively^{17, 18}, and those with 5 mM Ca^{2+} were of the order of magnitude of 10^{-5} M and 0.5–0.6 unit, respectively (T. NAKATA, unpublished observation).

Fig. 10 shows the pH-activity relationship of DTP hydrolysis by heavy meromyosin with that of ATP hydrolysis as a reference. The pH dependency of DTP hydrolysis was similar to that of ATP hydrolysis, i.e. a maximum at around pH 6–7 and a minimum at around pH 8–9 were observed with DTP.

Dependence of the v_{\max} of DTP hydrolysis on temperature was measured in the presence of 4.85 mM CaCl_2 and 0.5 M KCl at pH 7.6. As shown in Fig. 11, the temperature dependence gave a biphasic plot curving sharply near 14°. The activation energies were obtained as 12 and 34 kcal/mole above and below 14°, respectively.

Fluorescence of the DTP-heavy meromyosin system

Fluorescence excitation and emission spectra were measured before and after the addition of heavy meromyosin in a final concentration of 0.39 mg/ml to the

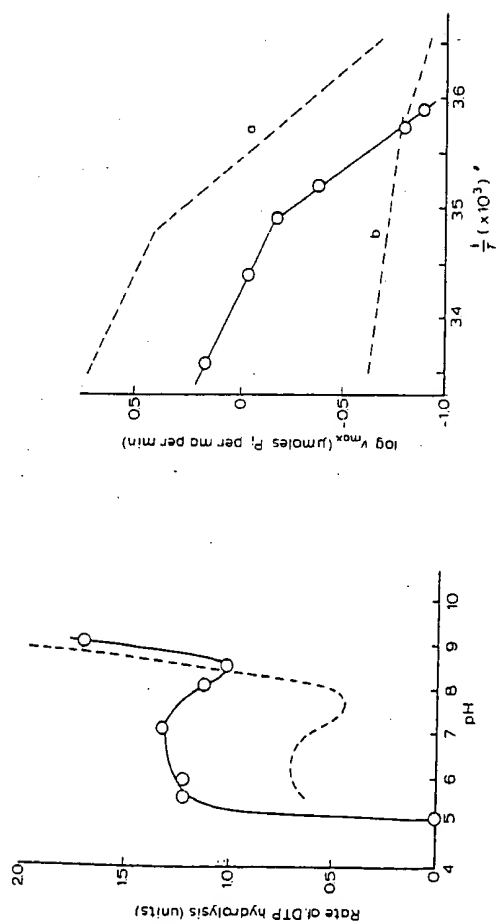


Fig. 10. pH-activity relationship of DTP hydrolysis by heavy meromyosin. Reaction mixture consisted of 0.1 mg/ml heavy meromyosin, 0.3 mM DTP, 0.5 M KCl, 4.85 mM CaCl_2 , and 20 mM Tris-maleate buffer (pH 5.1–7.3) or 20 mM Tris-HCl buffer (pH 7.9–9.1). Temperature, 25°. The broken line shows the pH-activity curve of ATP hydrolysis as a reference under the same conditions.

Fig. 11. Temperature dependency of the v_{\max} of DTP hydrolysis by heavy meromyosin. The v_{\max} was measured using a reaction mixture of 0.05 mg/ml heavy meromyosin, 0.5 M KCl, 4.85 mM CaCl_2 , 20 mM Tris-HCl buffer (pH 7.6) and various concentrations of DTP, at temperatures from 6 to 25°. Data of ATP hydrolysis (a) and ATP hydrolysis (b) were taken from the results of AZUMA AND TONOMURA²¹ obtained in 0.6 M KCl, 7 mM CaCl_2 , and 25 mM Tris-HCl buffer (pH 7.05).

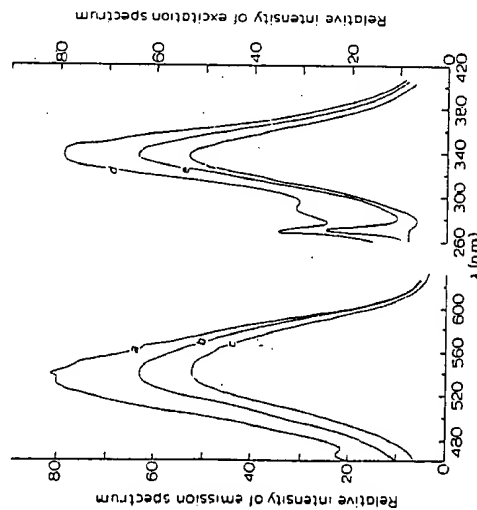


Fig. 12. Fluorescence excitation and emission spectra of DTP obtained after addition of heavy meromyosin. a, b, c, emission spectra, excitation at 340 nm. d, e, f, excitation spectra, emission at 537 nm. Reaction mixture consisted of 0.39 mg/ml heavy meromyosin, 7.95 μM DTP, 83.4 mM MgCl_2 , and 33.3 mM Tris-HCl buffer (pH 7.6). Temperature, 10°. Time elapsed until the measurement of the maximum of each spectrum after addition of heavy meromyosin: a, 20 sec; b, 90 sec; c, 160 sec; d, 33 sec; e, 89 sec; f, 159 sec.

reaction mixture containing 2-(dansylamino)ethanol, DDP or DTP in the presence of 83.4 mM MgCl_2 and 33.3 mM Tris-HCl buffer (pH 7.6), at 10°. The molar ratio of the added 2-(dansylamino)ethanol, DDP or DTP, to heavy meromyosin was nearly 7. The fluorescence excitation spectrum was obtained by the measurement of fluorescence at 537 nm, and the fluorescence emission spectrum was obtained by excitation at 340 nm.

The excitation and emission intensities increased a little with the addition of heavy meromyosin to 2-(dansylamino)ethanol (7.1 μM) or DDP (6.8 μM). Fig. 12 shows the fluorescence excitation and emission spectra obtained by the addition of heavy meromyosin to DTP (8.0 μM). In contrast to the above experiments using 2-(dansylamino)ethanol or DDP, an enhancement of about 60% in these intensities was observed immediately after the addition of heavy meromyosin to DTP, followed by a rapid decrease to the level obtained in the presence of DDP. The peak of the emission spectrum shifted from 540 to about 530 nm along with the increase in intensity.

The fluorescence excitation spectrum measured 33 sec after the addition of heavy meromyosin to DTP, clearly showed a peak at 290 nm which was not observed at 89 sec. When DDP (6.8 μM) or 2-(dansylamino)ethanol (7.1 μM) was present in place of DTP, the peak at 290 nm was not observed. The intensity at 290 nm was measured continuously after addition of heavy meromyosin in a final concentration of 0.39 mg/ml to various concentrations of DTP in the presence of 83.4 mM MgCl_2 and 0.5 M KCl at 16°. The first measurement was made 3–5 sec after the addition of heavy meromyosin. When the concentration of DTP was more than 19.8 μM , a plateau of intensity at 290 nm was observed which gradually decreased to a certain low level. The half-life of the decrease in intensity was obtained from a series of experiments as 26, 48, 62, or 102 sec with 8.0, 19.8, 31.8, or 39.8 μM DTP, respectively.

DISCUSSION

The molecular structure of DTP is shown in Fig. 13, together with that of ATP. The sizes of the compounds appear to be very close to one another and the similarity implies that DTP could be a substrate analog of ATP.

Like ATP, DTP was actually hydrolyzed easily by heavy meromyosin, the competition between ATP and DTP in the heavy meromyosin-ATPase reaction indicates that DTP binds to the ATP binding site (Fig. 8). DTP was not hydrolyzed by heavy meromyosin in the presence of 10 mM EDTA and 0.5 M KCl, while ATP hydrolysis was activated by EDTA. The nucleotide polyphosphates tested so far show a marked difference in their rates of hydrolysis depending on the structure of the purine or pyrimidine ring, those possessing an amino group in the 6-position being highly preferable for hydrolysis in the presence of EDTA¹⁹. The marked inhibition of DTP hydrolysis by EDTA might therefore be due to the lack of any amino group in the naphthalene ring in DTP.

The dansyl group is known as a fluorescent reagent, but it is also useful as an indicator of a hydrophobic environment. The addition of an organic solvent (ethanol) or bovine serum albumin to 2-(dansylamino)ethanol increased the fluorescence intensity and induced the blue shift of the fluorescence emission spectrum. When heavy meromyosin was added to 2-(dansylamino)ethanol, DDP or DTP, increase

in intensity and the blue shift in the fluorescence spectrum of the dansyl group were also observed. The increment of fluorescence intensity obtained with DTP was much higher than that obtained with other compounds, and the fluorescence intensity once increased with DTP decreased to the level of a mixture of DDP and heavy meromyosin after the hydrolysis of DTP to DDP. Since it was indicated that DTP binds to the ATP binding site, DTP is expected to be a hydrophobic probe of the active site of heavy meromyosin.

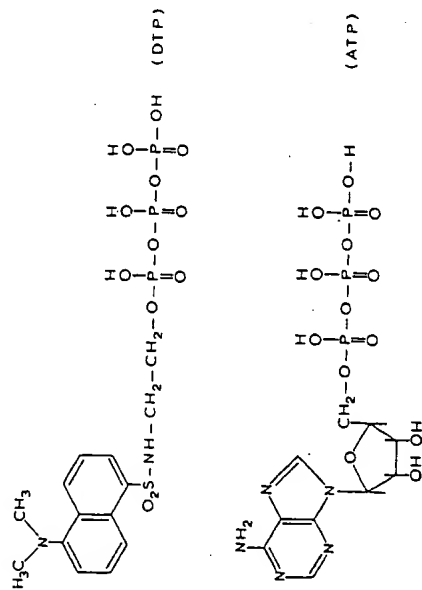


Fig. 13. Molecular structures of DTP and ATP.

CHEUNG AND MORALES²⁰ have shown that the maximum binding of 8-aniline-1-naphthalene sulfonate to myosin was obtained as 2 (mole/mole) and that the binding sites were located towards the head region of the myosin molecule. The results suggest that the hydrophobic region of the myosin molecule is very restricted and that the noncovalent binding of dansyl compounds to heavy meromyosin would also be limited.

The maxima of absorption and fluorescence emission spectra of DTP were at 328 and 534 nm, respectively. The absorption of DTP overlaps with the fluorescence of tyrosine and tryptophan. Energy transfer from the protein chromophore (tyrosine and/or tryptophan) to the dansyl group of DTP can therefore be assumed to occur. When heavy meromyosin was added to DTP, a particular excitation spectrum that rapidly disappeared was observed around 290 nm. The result shows that the excitation spectrum around 290 nm may be related directly to the energy transfer from the protein chromophore to DTP and that DDP is no longer able to participate in energy transfer after DTP hydrolysis. The rate constant of the decay of fluorescence intensity at the 290 nm peak was 0.06 unit in the presence of 100 mM MgCl₂ and 0.5 M KCl at 16° using CHANCE's²¹ formula. This value was similar to the rate constant of DTP hydrolysis by heavy meromyosin under the same conditions.

According to MORITA¹⁷, a red shift of the absorption spectrum around 290 nm is observed accompanied with the formation of Michaelis complex of heavy meromyosin. This shift is decreased by the hydrolysis of ATP to ADP. She explained the phenomenon of red shift by the displacement of tryptophan and tyrosine to the hydrophobic region. Binding of DTP to the active site might cause a displacement

DTP: A FLUORESCENT SUBSTRATE OF MYOSIN-ATPase

of the tryptophan, which moves by the binding of ATP, and the tryptophan probably participates in the energy transfer to the dansyl group of bound DTP.

ACKNOWLEDGMENT

We are greatly indebted to Dr. T. Ueda (Hokkaido Univ.) for guidance in the synthesis of DTP, and also to Mr. S. Shimokawa for the measurements of NMR spectra.

This work was supported by grants from the Muscular Dystrophy Associations of America, and from the Ministry of Education.

REFERENCES

- 1 Y. TONOMURA, K. INAMURA, M. Ikehara, H. UNO AND F. HARADA, *J. Biochem. Tokyo*, **61** (1967) 460.
- 2 T. P. SINGER AND E. S. G. BARRON, *Proc. Soc. Exp. Biol. Med.*, **56** (1944) 129.
- 3 W. W. KIELLEY AND L. B. BRADLEY, *J. Biol. Chem.*, **218** (1956) 653.
- 4 A. J. MURPHY AND M. F. MORALES, *Biochemistry*, **9** (1970) 1528.
- 5 L. GERSHMAN, A. STRACHER AND P. DREIZEN, *J. Biol. Chem.*, **244** (1969) 2726.
- 6 D. A. DERANLEAU AND H. NEURATH, *Biochemistry*, **5** (1966) 1413.
- 7 S. A. LATT, D. S. AULD AND B. L. VALLEE, *Proc. Natl. Acad. Sci. U.S.A.*, **67** (1970) 1383.
- 8 M. ONODERA AND K. YAGI, *Abstr. Annu. Meeting Biochem. Soc. Japan, Hiroshima*, **41** (1969) 584.
- 9 M. ONODERA AND K. YAGI, *Abstr. Annu. Meeting Biochem. Soc. Japan, Tokyo*, **42** (1970) 509.
- 10 A. G. SZENT-GYORGYI, *J. Mol. Biol.*, **2** (1960) 133.
- 11 C. H. FISKE AND Y. SUBBAROW, *J. Biol. Chem.*, **66** (1925) 375.
- 12 J. B. MARTIN AND D. M. DOTY, *Ann. Chem.*, **21** (1949) 965.
- 13 R. J. L. ALLEN, *Biochem. J.*, **34** (1940) 858.
- 14 N. SEILER AND M. WIECHMANN, *Z. Anal. Chem.*, **220** (1966) 109.
- 15 M. SMITH AND H. G. KHORANA, *J. Am. Chem. Soc.*, **80** (1958) 1141.
- 16 TH. FÖRSTER, *Fluoreszenz Organischer Verbindungen*, Vandenhoeck und Ruprecht, Göttingen, 1951, p. 85.
- 17 F. MORITA, *J. Biol. Chem.*, **242** (1967) 4501.
- 18 F. MORITA, *Biochim. Biophys. Acta*, **172** (1969) 319.
- 19 W. W. KIELLEY, in P. D. BOYER, H. LARDY AND K. MYRBACK, *The Enzymes*, Vol. 5, Academic Press, New York, 2nd ed., 1961, p. 162.
- 20 H. C. CHEUNG AND M. F. MORALES, *Biochemistry*, **8** (1969) 2177.
- 21 B. CHANCE, *J. Biol. Chem.*, **151** (1943) 553.
- 22 N. AZUMA AND Y. TONOMURA, *Biochim. Biophys. Acta*, **73** (1963) 499.

SYNTHESIS AND PROPERTIES OF A NEW FLUORESCENT ANALOG OF ATP:
ADENOSINE-5'-TRIPHOSPHORO- γ -1-(5-SULFONIC ACID) NAPHTHYLAMIDATE

LYNWOOD R. YARBROUGH

DEPARTMENT OF BIOCHEMISTRY, KANSAS UNIVERSITY MEDICAL CENTER

KANSAS CITY, KANSAS 66103

Received December 27, 1977

SUMMARY

An analog of ATP has been synthesized which contains the fluorophore, 1-aminonaphthalene-5-sulfonate attached via a γ -phosphoamidate bond. This analog is strongly fluorescent (quantum yield = 0.63) with an emission maximum at 460 nm; the excited state lifetime is 20 nsec. It is a substrate for DNA-dependent RNA polymerase of *E. coli* and wheat germ RNA polymerase II. It is also a substrate for *E. coli* valyl t-RNA synthetase, venom phosphodiesterase, and potato apyrase. Cleavage of the α - β phosphoryl bond as a result of RNA synthesis or by venom phosphodiesterase produces a 15 nm red shift in the fluorescence emission spectrum. This property should make this nucleotide useful for studies of the mechanisms of enzymatic reactions involving cleavage of the α - β phosphoryl bond.

INTRODUCTION

Nucleotides play important roles in many biological processes. These processes include DNA and RNA synthesis, protein synthesis, and energy transduction. A number of nucleotide analogs have been synthesized and used to study the role of nucleotides in various systems (1). These analogs have included those with altered chemical reactivity (AMPPnP and ATP- γ -S), altered chromophoric ring structures (6-thioguanosine triphosphate), and fluorescent derivatives such as ϵ -ATP and formycin triphosphate (2-7).¹

The use of fluorescent nucleotides, ϵ -ATP and formycin triphosphate, has often been limited by the properties of the enzyme or by the spectroscopic properties of the nucleotide. For example, ϵ -ATP contains a bridge group which prevents normal hydrogen bonding of the purine ring. This analog is

¹Abbreviations used are: (γ -AmNS)-ATP, adenosine-5'-triphosphoro- γ -1-(5-sulfonic acid) naphthylamidate; AMPPnP, adenylyl-imidodiphosphate; ATP- γ -S, adenosine-5'-O-(3-thiotriphosphate); and ϵ -ATP, 1,N⁶-ethenoadenosine triphosphate.

neither a substrate nor inhibitor for DNA-dependent RNA polymerase of *E. coli* an enzyme in which hydrogen bonding to the template evidently plays a key role (L. Yarbrough, unpublished observations). Formycin triphosphate is a substrate for DNA-dependent RNA polymerase, however, its excited state lifetime is only about 1 nsec and its quantum yield is very low ($Q = 0.054$) thus its application has been somewhat limited.

Grachev and Zaychikov (7) have reported the synthesis of an ATP analog containing aniline bound to the γ -phosphate via a phosphoamidate linkage. This analog is a good substrate for DNA-dependent RNA polymerase of *E. coli*. This suggested that it should be possible to prepare the analogous derivative containing 1-aminonaphthalene-5-sulfonate. Here I report the synthesis of this fluorescent derivative of ATP, adenosine 5'-triphosphoryl-(5-sulfonic acid) naphthylamidate, (γ -AmNS)-ATP, and some of its spectroscopic and enzymatic properties.

MATERIALS AND METHODS

Chemicals - The following chemicals were purchased from the sources listed in parenthesis: ATP, grade 1 (Sigma), [^3H] ATP (New England Nuclear), poly d (A-T) (P and L Labs), 1-aminonaphthalene-5-sulfonate (Tridom), and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Pierce).

Enzymes - *E. coli* RNA polymerase was purified as described previously (8). Wheat germ RNA polymerase II was purified as described by Jendrisak and Burgess (9). Purified valyl t-RNA synthetase was a gift of Drs. Ann Collins and George Marchin, Kansas State University. *E. coli* alkaline phosphatase, adenylate kinase, and apyrase were from Sigma; Acetate kinase was from Boehringer; cAMP dependent protein kinase from bovine heart was a gift of Dr. Ora Rosen, Albert Einstein College of Medicine, N.Y. Venom phosphodiesterase was purified from crude venom of *Crotalus adamanteus* by incubation for 3 hr at 37° and pH 3.6.

Spectroscopic measurements - Absorption spectra were obtained with a Cary model 118-C recording spectrophotometer. Fluorescence measurements were made at 25° with a Perkin-Elmer MPF-44 recording fluorescence spectrophotometer equipped with a microprocessor corrected spectra attachment. Samples had an absorbance of < 0.1 absorbance unit to obviate significant inner filter effects. Excited state lifetime measurements were obtained with the Ortec model 9200 single photon counting system. Data was deconvoluted and fit to a single or double exponential by the method of moments.

Enzymatic Digestion of (γ -AmNS)-ATP - Reactions in 0.05 mL contained: 0.05 M Tris HCl, pH 8, 10^{-2} M MgCl_2 , 10^{-4} M dithiothreitol and 2 mM ATP or (γ -AmNS)-ATP. Samples were incubated at 37° for 3 hr with 25 μg of *Crotalus adamanteus* phosphodiesterase, 25 μg of *E. coli* alkaline phosphatase, or 25 μg of each.

Synthesis and Sp

synthesized from 1-am the water soluble car using a modification synthesis procedure w tion product, (γ -AmNS cellulose. The purif spot with a mobility ethyleneimine cellulo Incubation of (γ -AmNS complete disappearanc chromatographic speci with authentic ATP; t ted with 1-aminonaph

The absorption s a maximum at about 24 about 315 nm. Based tion coefficient at 3 lene-5-sulfonate show results are consisten 1-aminonaphthalene-5-s is also shown in Fig. Following acid hydroly to 330 nm, the same a fluorescence emission a broad maximum at 46 quinine sulfate in 0. excited state lifetime 1-aminonaphthalene-5-s conditions.

RESULTS

Synthesis and Spectroscopic Properties of (γ -AmNS)-ATP. (γ -AmNS)-ATP was synthesized from 1-aminonaphthalene-5-sulfonate, [^3H]ATP (500 cpm/nmole), and the water soluble carbodiimide, 1-ethyl-3-(3-dimethylamino propyl)carbodiimide, using a modification of the procedure of Babkina *et al.* (10). Details of the synthesis procedure will be presented in a subsequent communication. The reaction product, (γ -AmNS)-ATP (Fig. 1), was purified by chromatography on DEAE cellulose. The purified nucleotide showed a single intense blue fluorescent spot with a mobility about one-half that of ATP when chromatographed on polyethyleneimine cellulose according to the procedure of Gonzales and Geel (11). Incubation of (γ -AmNS)-ATP in 0.5 N HCl for 30 minutes at 37° resulted in the complete disappearance of the original material and the appearance of two new chromatographic species. One strongly absorbed ultraviolet light and migrated with authentic ATP; the other exhibited a yellow-green fluorescence and migrated with 1-aminonaphthalene-5-sulfonate.

The absorption spectrum of (γ -AmNS)-ATP is shown in Fig. 2. It exhibits a maximum at about 243 nm, a shoulder at 260 nm, and a broad band centered at about 315 nm. Based on radioactivity measurement of [^3H]ATP, a molar extinction coefficient at 315 nm of $5580 \text{ M}^{-1} \text{ cm}^{-1}$ can be calculated. 1-aminonaphthalene-5-sulfonate shows a similar band at about 330 nm with $\epsilon = 6000$. These results are consistent with a conjugate containing 1 mole of ATP and 1 mole of 1-aminonaphthalene-5-sulfonate. The corrected fluorescence excitation spectrum is also shown in Fig. 2. Excitation maxima are observed at 243 and 315 nm. Following acid hydrolysis, the long wave length excitation maximum is shifted to 330 nm, the same as found for 1-aminonaphthalene-5-sulfonate. The corrected fluorescence emission spectrum of (γ -AmNS)-ATP is shown in Fig. 3. It exhibits a broad maximum at 460 nm. The quantum yield was calculated to be 0.63 using quinine sulfate in 0.1 N H_2SO_4 as standard ($Q = 0.55$). Measurements of the excited state lifetime show a single component with a lifetime of 20 nsec. 1-aminonaphthalene-5-sulfonate shows a lifetime of about 5 nsec under the same conditions.

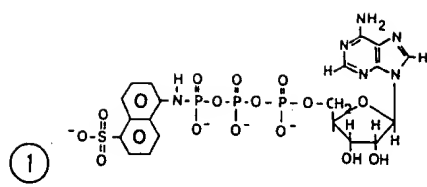


Fig. 1. Structure of (γ -AmNS)-ATP

Fig. 2. Absorption (---) and corrected fluorescence excitation (—) spectra of (γ -AmNS)-ATP. Measurements were performed at 25° in 0.05 M Tris.HCl, pH 8, 0.05 M NaCl, 10^{-2} M $MgCl_2$, 10^{-4} M EDTA. For the absorption spectrum the nucleotide concentration was 4.3×10^{-5} M. For the fluorescence excitation spectrum, the nucleotide concentration was 2×10^{-6} M. Emission was measured at 460 nm through a 350 nm cut-off filter.

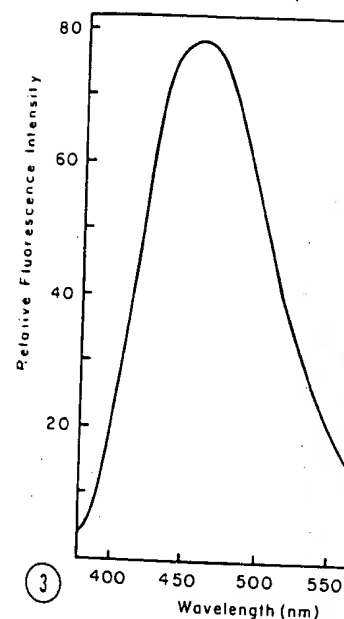
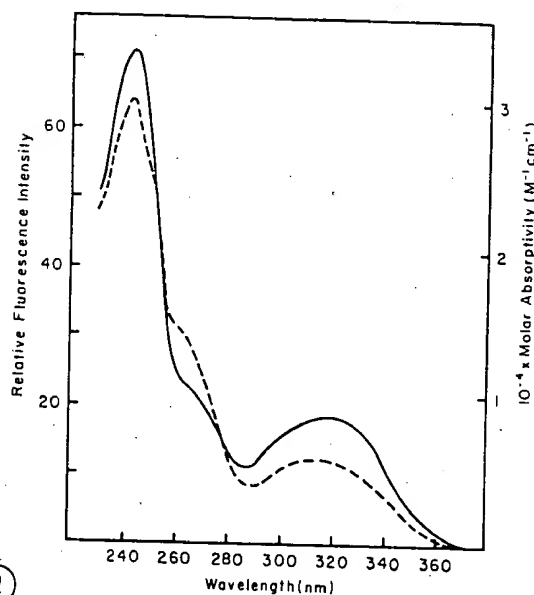


Fig. 3. Corrected fluorescence intensity. The conditions were the same as in Fig. 2. Emission was at 320 nm.

Fig. 4. Alterations in fluorescence intensity induced by enzymatic utilization of (γ -AmNS)-ATP.

Enzymatic Properties of (γ -AmNS)-ATP. The ability of (γ -AmNS)-ATP to substitute for ATP in a number of enzymatic reactions was examined. Table 1 shows that this nucleotide is a good substrate for DNA-dependent RNA polymerase isolated from *E. coli* and wheat germ (polymerase II). It is also a substrate for valyl t-RNA synthetase from *E. coli*. Thus this nucleotide appears to be an effective substrate for reactions involving cleavage of the α - β phosphoryl bond.

(γ -AmNS)-ATP is not a substrate under the conditions tested for any of the following kinases: acetate kinase, adenylate kinase, or cAMP-dependent protein kinase. It is degraded by potato apyrase and venom phosphodiesterase of *Crotalus adamanteus* but not by bacterial alkaline phosphatase.

The fluorescence peaks at 460 nm (curve A, Fig. 3) are broken. The fluorescence intensity at 460 nm (curve A, Fig. 3) is most of the nucleotide v (curve B, Fig. 4). The fluorescence intensity decreases to 10 nsec. A similar change in fluorescence intensity with venom phosphodiesterase. Although (γ -AmNS)-ATP alone, when the nucleotide is utilized by polymerase II and alkaline phosphatase at 520 nm, the same as found in Fig. 4). Analysis of the

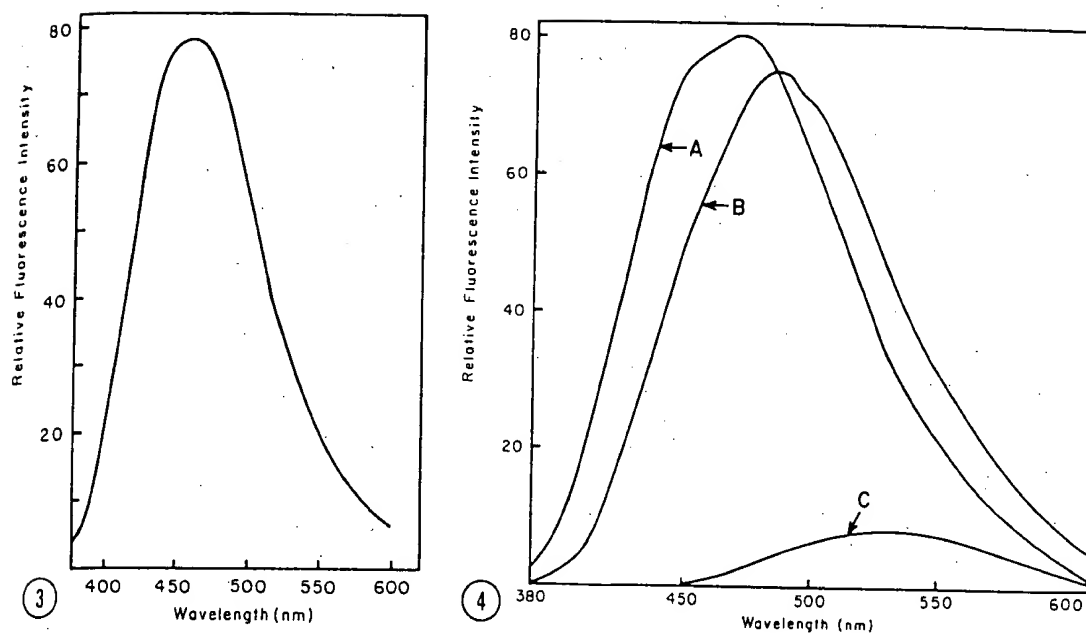


Fig. 3. Corrected fluorescence emission spectrum of (γ-AmNS)-ATP. Conditions were the same as in Fig. 2 except that no cut-off filter was used. Excitation was at 320 nm.



Fig. 4. Alterations in the fluorescence emission spectrum of (γ-AmNS)-ATP produced by enzymatic utilization. Measurements were performed as described in Fig. 3.

The fluorescence properties of (γ-AmNS)-ATP are altered when P-O or P-N bonds are broken. The intact nucleotide has a fluorescence emission maximum at 460 nm (curve A, Fig. 4). Following extensive RNA synthesis during which most of the nucleotide was utilized, the emission maximum shifted to 475 nm (curve B, Fig. 4). The excited state lifetime decreased from 20 nsec to 16 nsec. A similar change in fluorescence properties was produced by digestion with venom phosphodiesterase.

Although (γ-AmNS)-ATP is not digested by bacterial alkaline phosphatase alone, when the nucleotide is digested with a combination of venom phosphodiesterase and alkaline phosphatase, the fluorescence emission maximum shifts to 520 nm, the same as found for free 1-aminonaphthalene-5-sulfonate (curve C, Fig. 4). Analysis of the reaction products by thin layer chromatography

Table 1. Utilization of (γ -AmNS)-ATP by DNA-dependent RNA polymerase and valyl t-RNA synthetase.

Enzyme	Activity (%)	
	ATP	(γ -AmNS)-ATP
RNA polymerase (<i>E. coli</i>)	100	60
RNA polymerase II (wheat germ)	100	27
valyl t-RNA synthetase (<i>E. coli</i>)	100	20

Assays for RNA polymerase contained in 0.1 mL: 0.05 M Tris HCl, pH 8, 10^{-2} M $MgCl_2$, 10^{-3} M dithiothreitol, 10^{-4} M UTP, 10^{-4} M [3H]ATP (4980 cpm/nmole) or (γ -AmNS)[3H]ATP (500 cpm/nmole), 20 nmoles of poly d (A-T), and 8 pmoles of *E. coli* holo enzyme. Assays for wheat germ RNA polymerase contained 16 nmoles of denatured calf thymus DNA in lieu of poly d (A-T), 3 mM $MnCl_2$ instead of $MgCl_2$, [3H]GTP (11,300 cpm/nmole), 0.05 M $(NH_4)_2SO_4$, 5 μ g of purified wheat germ RNA polymerase, and other components as described above. Samples were incubated for 10 min at 37°, precipitated with 5% trichloroacetic acid, and the precipitate collected on glass fiber filters. The filters were dried and counted in a toluene based liquid scintillation fluid. Assays for valyl t-RNA synthetase contained in 0.1 mL: 0.1 M Tris.HCl, pH 7.3, 10^{-2} M $MgCl_2$, 10^{-2} M KCl, 10^{-4} M dithiothreitol, 10^{-4} M [3H]valine (35 cpm/pmole), 50 μ g t-RNA, 2 mM ATP or (γ -AmNS)-ATP and 0.1 μ g of purified *E. coli* valyl t-RNA synthetase. Following a 15 min incubation at 37°, samples were precipitated with 5% trichloroacetic acid, the precipitates collected, and counted by liquid scintillation as described above.

revealed the presence of two species. One absorbed u.v. light and migrated with adenosine; the other was fluorescent and migrated with 1-aminonaphthalene-5-sulfonate. No (γ -AmNS)-ATP was detected following digestion.

DISCUSSION

(γ -AmNS)-ATP should be an excellent probe for many ATP requiring enzymes, especially those which cleave the α - β phosphoryl bond such as nucleic acid polymerases and t-RNA synthetases. This nucleotide has an absorption band in the region 300-350 nm which permits its selective excitation. Since this is the region in which tryptophan fluorescence occurs, it is also a potential acceptor for resonance energy transfer from intrinsic fluorophores of proteins. In addition, the relatively long excited state lifetime makes it potentially useful for studies of fluorescence polarization. The quantum yield is high and

is altered when P-O

study the dynamics c

The reaction us

other nucleotide ana

parable GTP analog.

ribo- as well as de

It has not yet

incorporated into th

It appears that it c

the corresponding de

studies show that (γ

ics of RNA chain ini

This research w

Mid-America Cancer

the National Foundat

Association-Kansas A

for helpful suggesti

for expert technical

1. Young, R., Babc
10: 2484-2489.
2. Young, R. (197
3. Goody, R.S., an
4. Darlix, J., Fro
5. Secrist, J.A. I
6. Ward, D.C., Rei
7. Grachev, M. and
8. Yarbrough, L. ai
9. Jendrisak, J. ai
10. Babkina, G.T., ;
11. Gonzales, L.W. ;

ase and valyl

(%)

(γ -AmNS)-ATP

60

27

20

8, 10^{-2} M
nmole) or
pmoles of
d 16 nmoles
stead of
ied wheat
les were
id, and the
ed and
valyl t-RNA
s, 10^{-2} M
t-RNA, 2
nthease.
1 5% tri-
l scintil-

migrated with

lane-5-sul-

g enzymes,
c acid poly-
nd in the
s is the
al accep-
eins. In
ally use-
high and

is altered when P-O or P-N bonds are broken. This property may allow one to study the dynamics of the phosphoryl bond breaking step.

The reaction used to synthesize (γ -AmNS)-ATP can be used to synthesize other nucleotide analogs. For example, we have already synthesized the comparable GTP analog. In addition, it should be possible to synthesize other ribo- as well as deoxyribonucleoside mono, di, or triphosphate derivatives.

It has not yet been determined whether (γ -AmNS)-ATP is capable of being incorporated into the 5' terminus of RNA chains, i.e., acting as an initiator. It appears that it can however, since Grachev and Zaychikov (7) have shown that the corresponding derivative, ATP- γ -anilate, can initiate RNA chains. If studies show that (γ -AmNS) can initiate, we plan to use it to study the dynamics of RNA chain initiation.

ACKNOWLEDGEMENTS

This research was supported by a research and equipment grant from the Mid-America Cancer Center Project, a Basil O'Connor Starter Research Grant from the National Foundation-March of Dimes, and a grant from the American Heart Association-Kansas Affiliate. I thank Dr. P.T. Gilham, Purdue University, for helpful suggestions regarding the synthesis procedure and Mr. Mike Loggan for expert technical assistance.

REFERENCES

1. Young, R., Babcock, D., Ballantyne, W., and Ojala, D. (1971) *Biochemistry* 10: 2484-2489.
2. Young, R. (1975) *Adv. in Enz.* 43: 1-56, John Wiley, N.Y.
3. Goody, R.S., and Eckstein, F. (1971) *J. Am. Chem. Soc.* 93: 6252-6257.
4. Darlix, J., Fromageot, P., and Reich, E. (1973) *Biochemistry* 12: 914-919.
5. Secrist, J.A. III, Barrio, J., and Leonard, N.J. (1972) *Science* 175: 646-647.
6. Ward, D.C., Reich, E., and Stryer, L. (1969) *J. Biol. Chem.* 244: 1228-1237.
7. Grachev, M. and Zaychiov, E.F. (1974) *FEBS Lett.* 49: 163-166.
8. Yarbrough, L. and Hurwitz, J. (1974) *J. Biol. Chem.* 249: 5394-5399.
9. Jendrisak, J. and Burgess, R.R. (1975) *Biochemistry* 14: 4639-4645.
10. Babkina, G.T., Zarytova, V.F. and Knorre, D.G. (1975) *Biorg. Khim.* 1:611-615.
11. Gonzales, L.W. and Geel, S.E. (1975) *Anal. Biochem.* 63: 400-413.

Direct Observation of Complexes Formed between recA Protein and a Fluorescent Single-Stranded Deoxyribonucleic Acid Derivative¹

Marc S. Silver* and Alan R. Fersht*

ABSTRACT: The reaction of chloroacetaldehyde with single-stranded DNA (ssDNA) yields ϵ DNA, a highly fluorescent substance. The binding of recA protein to ϵ DNA nearly doubles its fluorescence yield. The enhanced fluorescence signals the formation of a recA- ϵ DNA complex. This complex exhibits an ATPase activity as great as that of the corresponding recA-ssDNA complex. Addition of a saturating concentration of adenosine 5'-O-(3-thiotriphosphate) (ATP γ S) to a solution of the recA- ϵ DNA complex yields a further rise in fluorescence. Saturation with ATP produces the same rise. The nucleotide triphosphates have converted the recA- ϵ DNA complex into the respective ATP γ S-recA- ϵ DNA and ATP-recA- ϵ DNA complexes. The fluorescence changes that ac-

company the formation of the three complexes have enabled us to (1) establish by titration that recA protein binds to 6.0 ± 0.3 nucleotides of ϵ DNA, (2) show that the binding of ATP to the recA- ϵ DNA complex is highly cooperative under various conditions, with a Hill coefficient of 2.4-4.9 and $K_{app} = 25 \pm 2 \mu\text{M}$, (3) show that the binding of ATP γ S is also highly cooperative, with a Hill coefficient of 3.3-4.2 and $K_{app} \approx 0.5 \mu\text{M}$, and (4) perform initial measurements on the rate at which recA protein transfers between polynucleotides. The experiments provide the first direct observation of an ATP-recA-ssDNA-like complex, and they illuminate some of the properties of such complexes.

The recA protein shows a remarkable range of activities for so small a molecule. Particularly interesting is its ability to catalyze ATP-dependent DNA strand assimilation. Studies with the recA protein are consequently providing important insights into genetic recombination mechanisms (Geider & Hoffmann-Berling, 1981; Radding, 1981). In particular, the structural features that homologous DNA molecules must possess if they are to undergo recA-promoted¹ strand assimilation are being explored (West et al., 1981; DasGupta & Radding, 1982). Complementary biochemical studies have broadly established the nature of the interactions between recA protein and polynucleotides and nucleotide triphosphates and the factors controlling the ability of recA protein to act as a protease (Weinstock et al., 1981a-c; Craig & Roberts, 1980). These experiments rely primarily on selective filter-binding assays to characterize the various recA-DNA complexes presumed to be involved. Data obtained with the ultracentrifuge and electron microscope provide supplementary information. A spectroscopic method that allows recA-catalyzed reactions to be continuously monitored would make it possible to examine more closely the nature of these complexes and the dynamics governing their formation and disappearance. We describe here such a method and the initial results it has yielded.

The essential reagent is ϵ DNA, obtained by treating ssDNA with chloroacetaldehyde. This highly fluorescent modification of ssDNA has been known for nearly 10 years, but its great potential for investigating protein-ssDNA interactions does not appear to have been realized (Lee & Wetmur, 1973). The corresponding derivative of poly(rA), poly(ϵ A), has been employed by several investigators (Ledneva et al., 1978; Toulmé & Hélène, 1980) but, as we shall see, is of no use in the recA protein system. The chloroacetaldehyde reaction converts adenosine to 1,N⁶-ethenoadenosine and cytidine to 3,N⁴-ethenocytidine. It is the high fluorescence of the former at

neutral pH ($\lambda_{max} \sim 405 \text{ nm}$) that renders ϵ DNA and poly(ϵ A) so useful (Leonard & Tolman, 1975).

Experimental Procedures

Materials

Commercial samples of ADP (Sigma), ATP (Sigma), and ATP γ S (Boehringer) were used as received. Highly polymerized calf thymus dsDNA (Sigma) was converted to ssDNA by heating it for 20 min at 100 °C and then plunging it into an ice bath. The concentrations of all these nucleic acid derivatives were determined spectroscopically (Weinstock et al., 1981a). Thin-layer chromatography established that the ATP γ S contained $25 \pm 5\%$ ADP, and all cited concentrations for ATP γ S allow for this. Other purchased chemicals were of the highest purity available.

In all but two experiments, a single stock recA protein preparation was employed. It had been purified by Sephacryl S-300 filtration and is certainly >98% pure and binds 1.0 mol of nucleotide/mol of recA monomer (Cotterill et al., 1982). recA protein concentrations were determined spectrophotometrically (Cotterill et al., 1982).

Our procedure for synthesizing ϵ DNA was based primarily on an earlier method for preparing poly(ϵ A) from poly(rA) (Steiner et al., 1973). Chloroacetaldehyde was obtained by heating a mixture of 24 mL of $\text{CH}_2\text{ClCH}(\text{OCH}_3)_2$ (Aldrich), 10 mL of H_2SO_4 , and 250 mL of water under reflux for 20 min and distilling the resultant homogeneous solution until $\sim 130 \text{ mL}$ of distillate had been collected. This distillate, when diluted to a volume of 150 mL, had a pH of 5 ± 0.5 . It was redistilled until $\sim 100 \text{ mL}$ of distillate had been collected. The stock chloroacetaldehyde solution was obtained by diluting the second distillate to 130 mL. The principle synthesis of ϵ DNA began with the addition of 29 mL of the chloroacetaldehyde

¹ From the Department of Chemistry, Imperial College of Science and Technology, London SW7 2AY, U.K. Received April 16, 1982. This work was supported by SERC Grant GR/A94249.

* Permanent address: Chemistry Department, Amherst College, Amherst, MA 01002.

¹ Abbreviations: ss, single stranded; ds, double stranded; ATP γ S, adenosine 5'-O-(3-thiotriphosphate); NTP, nucleoside triphosphate; poly(ϵ A), poly(1,N⁶-ethenoadenylic acid); ϵ DNA, product obtained by treating ssDNA with chloroacetaldehyde, which contains 1,N⁶-ethenoadenosine and 3,N⁴-ethenocytidine residues; recA, recA protein; Tris, tris(hydroxymethyl)aminomethane.

reagent to a solution prepared from 3 μ g of ssDNA, 6 mL of 1.7 M sodium acetate buffer (pH 5.2) and 24 mL of water. Reaction was allowed to continue at 40 °C for 280 min. The pH of the mixture was raised from 4.9 to 7.1 by the careful addition of NaOH and the ϵ -DNA isolated by ethanol precipitation. Several redissolutions and reprecipitations served to purify the product (Steiner et al., 1973). The solution obtained prior to the final precipitation was incubated at 40 °C overnight to hasten the decomposition of undesirable reaction intermediates that may have accumulated (Krzyzusiak et al., 1981). About 20 mg of colorless ϵ -DNA resulted. Aqueous solutions of this material have shown no detectable change in spectroscopic properties during a 3-month period.

The concentration of ϵ -DNA was determined by phosphate analysis (Ames, 1966). In standard buffer, our material showed λ_{\min} 244 nm ($\epsilon = 4700$), a broad maximum at 250–267 nm ($\epsilon_{260} = 6300$), and $\epsilon_{300} = 970$. Although it is not important to know the extent of modification in the ϵ -DNA, the following procedure suggests that $75 \pm 5\%$ of the adenine and cytosine rings have been converted to the etheno derivatives [cf. Lee & Wetmur (1973)]. In a trial run, ssDNA was treated with chloroacetaldehyde overnight. The slightly yellow product isolated, assumed to be 100% modified, showed λ_{\min} 247 nm and $OD_{270}/OD_{260} = 1.03$. The corresponding numbers for the starting material are 230 nm and 0.83. By interpolation, the ϵ -DNA used in our experiments is 82% or 70% modified, if the changes described depend linearly on the extent of modification.

Poly(ϵ A) was similarly prepared from poly(rA). Its spectroscopic properties were in excellent agreement with those reported, and its concentration was determined as described elsewhere (Ledneva et al., 1978).

Methods

All fluorescence experiments were performed with a Perkin-Elmer MPF-44B instrument operated in the ratio mode, generally with $\lambda_{\text{ex}} = 300$ nm and $\lambda_{\text{em}} = 400$ nm. A cuvette of approximately 1.5-mL capacity was positioned in a thermostated cell holder; it was illuminated over a 0.4-cm path length. The absorbance of the solutions employed was almost always <0.02 , but at the very end of some titrations, it rose to ~ 0.025 .

All experiments were performed at 25 °C and, except when noted, in standard buffer, consisting of 20 mM Tris-HCl, pH 7.5, containing 10 mM $MgCl_2$ and 1 mM dithiothreitol. A typical run involved the addition of small volumes of the reactants to 1.0 mL of standard buffer. Solutions were mixed by gently inverting the stoppered cell several times. *recA* protein was handled either with plastic apparatus or with glassware that had been previously treated with dimethyldichlorosilane. However, Hamilton syringes used for the addition of *recA* protein in some titration experiments were merely rinsed with the *recA* protein solution prior to use.

Miscellaneous Points. (1) The fluorescence titrations for determining the stoichiometry for the binding of ATP γ S to the *recA*- ϵ -DNA complex were performed on 200 μ L of solution in a cuvette of ~ 300 - μ L capacity. The spectrophotometer was set at $\lambda_{\text{ex}} = 310$ nm and $\lambda_{\text{em}} = 450$ nm. (2) ATPase experiments, performed with, [γ - 32 P]ATP (Amersham), determined the rate of release of radioactivity that was not adsorbed to activated charcoal. (3) In some experiments reported here and other unpublished ones, the order of mixing of reagents significantly affects what is observed. We have specified the order followed where that is a consideration. (4) All polynucleotide concentrations are reported as nucleotide residues.

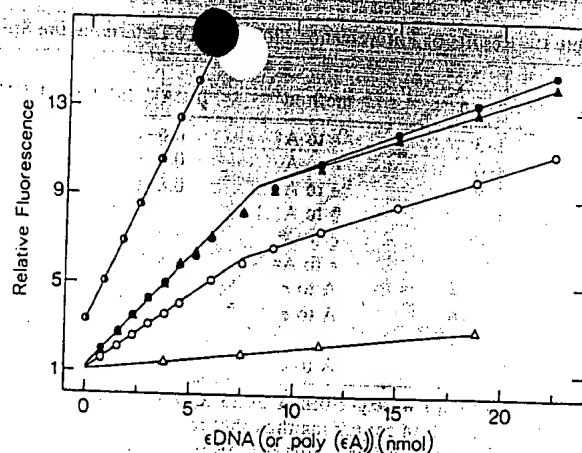


FIGURE 1: Titration of 1.24 nmol of *recA* protein at 25 °C in standard buffer. Aliquots of 750 μ M ϵ -DNA solution were added to approximately 1 mL of the *recA* protein solution which also held 930 μ M ATP (●), 140 μ M ATP γ S (▲), or no NTP (○). The two controls illustrate the addition of ϵ -DNA to 1.0 nmol of α -chymotrypsin in the presence of 140 μ M ATP γ S (△) and the attempted titration of 0.6 nmol of *recA* protein with poly(ϵ A) (○). The latter curve has been rescaled in order to fit it on the plot. It shows no change in slope in the region of 3.7 nmol of poly(ϵ A).

Results

Two critical observations lie at the heart of all that follows. First, addition of *recA* protein to a solution of ϵ -DNA greatly enhances the fluorescence of ϵ -DNA at 400 nm. For example, the fluorescence yield from a mixture of 0.6 μ M *recA* protein with 6.4 μ M ϵ -DNA in standard buffer at 25 °C is $\sim 80\%$ higher than the sum of the separated individual components. The complex responsible for this enhanced fluorescence will be designated as the *recA*- ϵ -DNA complex. Second, addition of saturating concentrations of ATP (≥ 500 μ M) or ATP γ S (≥ 30 μ M) to the solution of *recA*- ϵ -DNA complex formed in the preceding experiment causes a further substantial rise in fluorescence. Under any particular set of conditions, rises in fluorescence for the two NTP's are identical. The experiments described below examine the utility of these fluorescence changes for exploring the behavior of *recA* protein.

Fluorescence Titrations. The expectation that *recA* protein binds strongly to ϵ -DNA suggests performing fluorescence titrations to define accurately the number of nucleotides covered by a *recA* monomer (Toulmé & Hélène, 1980). Two procedures are possible, for ϵ -DNA can be added to *recA* protein, or vice versa. Since a titration may be performed in the presence of saturating concentrations of ATP or ATP γ S or in the absence of any NTP, a total of six kinds of titrations may be attempted. All have been tried. Figures 1 and 2 give one example of each kind and illustrate a few control experiments. The difference in slope between the initial and final segments of each titration is greater in Figure 2 than it is for the corresponding titration in Figure 1 because the contribution of the fluorescence at 400 nm from excess *recA* protein, which the final portion of Figure 2 reflects, is so modest.

The equivalence point for each titration was obtained by determining the point of intersection of the least-squares straight lines passed through its initial and terminal phases. Table I summarizes the results obtained.

Binding of ATP and ATP γ S to the *recA*- ϵ -DNA Complex. As previously stated, addition of a high concentration of ATP or ATP γ S to a solution of the *recA*- ϵ -DNA complex affords a substantial fluorescence enhancement. Figure 3 graphically demonstrates this effect for ATP. Saturating concentrations of the two nucleotides produce the same enhancement, within experimental error.

Table 1. Results of Fluorescence Titration

run	method ^b	[recA] ₀ (μM)	[εDNA] ₀ (μM)	[ATP] ₀ (mM)	[ATPγS] ₀ (μM)	nucleotides per recA monomer ^c
1-3	ε to A	0.6-1.2				
4-6	ε to A	0.6-1.2				
7-9	ε to A	0.6-1.2		0.93-1.4		5.7 ± 0.1
1-9	ε to A				60-140	6.0 ± 0.1
10 ^d	ε to A	1.2				6.3 ± 0.1
11 ^d	ε to A	0.6			60	6.0 ± 0.2
12-14	A to ε		3.7-7.4		35	5.9 ± 0.1
15-16	A to ε		3.7-7.4			6.4 ± 0.1
17-21 ^e	A to ε		1.4-7.4	0.78-1.85		7.3 ± 0.1
12-21	A to ε				30-140	7.7 ± 0.1
						8.9 ± 0.2
						8.3 ± 0.3

^a All experiments were performed at 25 °C in standard buffer. Small aliquots of either 750 μM εDNA or 124 μM recA protein were added to ~1 mL of solution containing the other reagents at the stated concentrations. ^b ε to A signifies addition of εDNA; A to ε, addition of recA protein. ^c The average value for this ratio at the equivalence point for the indicated runs. Figures 1 and 2 illustrate the experiments from which these data derive. Standard errors are also given. ^d Runs 10 and 11 employed a second (190 μM) and third (122 μM) batch of εDNA were titrated with 24.8 μM recA protein. ^e The two runs with 1.4 μM

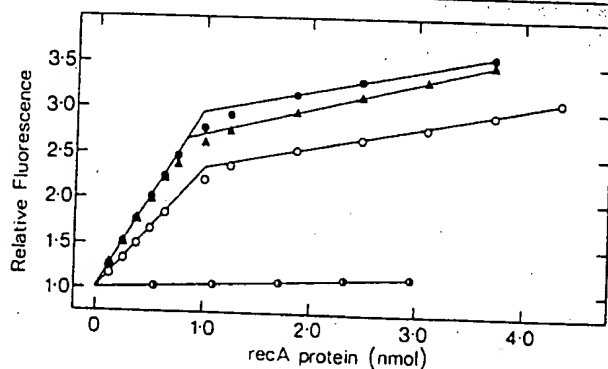


FIGURE 2: Titration of 7.5 nmol of εDNA at 25 °C in standard buffer. Aliquots of 124 μM recA protein solution were added to about 1 mL of the εDNA solution in the presence or absence of NTP. The symbols (●), (▲), and (○) have the same meaning as in Figure 1. The control (○) describes the addition of recA protein to a solution holding 4.4 nmol of poly(εA) and 57 μM ATPγS. It shows no detectable change in slope. The slope is so slight because the added recA protein barely perturbs the intensity of the highly fluorescent poly(εA) solution.

These factors give the titration curves in Figures 1 and 2 their characteristic forms. In each figure, the two titrations that include an NTP give a sharper change near the equivalence point than does the one without. The initial rise in fluorescence for the latter reflects solely the fluorescence enhancement associated with recA-εDNA complex formation. With the other two, we see this enhancement reinforced by the contribution from the conversion of that complex into what is most conveniently designated an ATP-recA-εDNA or ATPγS-recA-εDNA complex. Furthermore, the titration curves in the presence of ATP and ATPγS are nearly superimposable in Figure 1 and match closely in Figure 2. The following two qualitative observations on the nature of the NTP-recA-εDNA complexes are worth noting: (1) addition of ADP to a solution of recA-εDNA complex results in a slight decrease in the measured fluorescence; (2) addition of either NTP to a solution of recA-εDNA complex does not affect the measured intensity if Ca²⁺ is substituted for Mg²⁺ in the standard buffer or if no divalent cation is present.

When less than a saturating concentration of ATP (25-60 μM) is mixed with preformed recA-εDNA complex, the measured fluorescence changes in a characteristic way. As Figure 3 shows, it rapidly rises to a maximum value, remains constant for 0.5-3 min, and then commences a slow descent. If allowed to proceed long enough, the runs show a final fluorescence that generally lies slightly below the original level. The difference between the highest value reached and the original one, ΔF, is greater when a larger ATP concentration

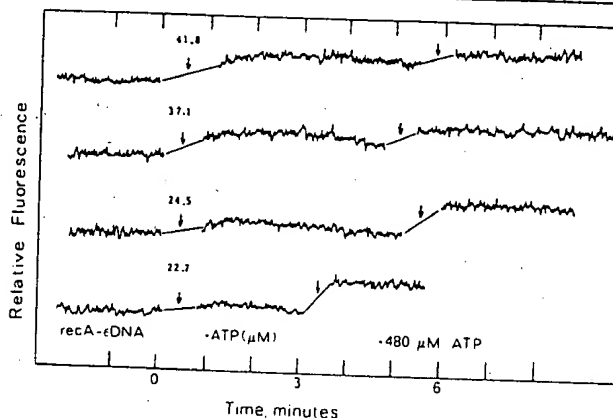


FIGURE 3: Fluorescence changes that characterize the binding of ATP to the recA-εDNA complex. The extreme left of each run represents the fluorescence of 7.4 μM εDNA plus 0.61 μM recA protein in standard buffer (this was the same for each run, but the traces have been displaced vertically). At the first arrow, [ATP] was increased to the level shown; at the second, it was increased to >480 μM. Traces of actual recordings obtained are shown, but the straight segments represent periods when the pen returned to zero as a reagent was added (the same holds for Figure 6). The final fluorescence intensity average was 1.16 ± 0.01 relative to the initial value for 13 runs in this experiment.

is introduced. The observations suggest a method for determining the apparent dissociation constant, K , and the degree of cooperativity characterizing the ability of ATP to convert the recA-εDNA complex into the ATP-recA-εDNA one.

The experiments are best done by preparing a stock solution containing recA protein and εDNA at the desired concentrations. The following three-step procedure is used for each data point (see Figure 3): (1) record the fluorescence of 1.0 mL of the stock; (2) add 0.5-5 μL of a relatively dilute ATP solution and record the time-dependent fluorescence change, thus determining ΔF; and (3) after the measured fluorescence has peaked, add 5-10 μL of a second ATP solution that is sufficiently concentrated to convert all the recA-εDNA complex into ATP-recA-εDNA. The difference between the final, constant fluorescence and that in step 1 defines ΔF_m.

Since the effect of [ATP] upon the ATPase activity of the recA-ssDNA complex has been treated successfully in terms of the Hill equation (Weinstock et al., 1981b), the same approach has been attempted for the fluorescence experiments. The data for each set of runs have been plotted according to the appropriate form of the Hill equation:

$$\log [\Delta F / (\Delta F_m - \Delta F)] = h \log [\text{ATP}] - \log K \quad (1)$$

As Figure 4 illustrates, each plot displays satisfactory linearity.

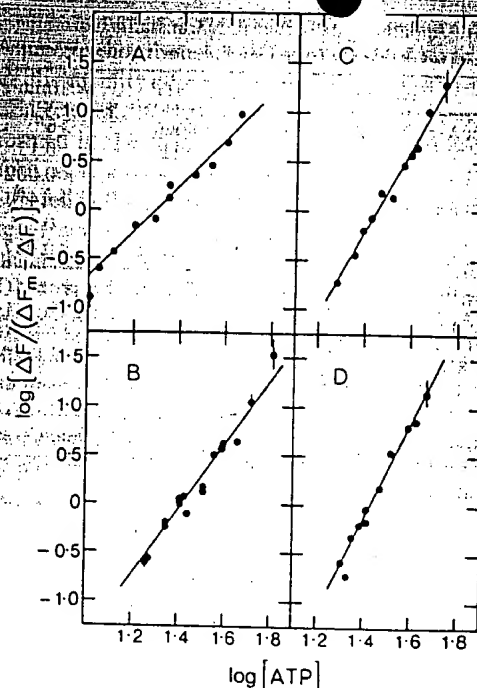


FIGURE 4: Binding of ATP to the recA-εDNA complex under standard conditions. The data are plotted according to the Hill equation (eq 1) with [ATP] values expressed in micromolar: (A) 1.2 μM recA protein, [εDNA]/[recA] = 3.1; (B-D) 0.61 μM recA protein and [εDNA]/[recA] = 6.1, 12, and 18, respectively.

Table II: Cooperative Binding of ATP and ATPγS to recA-εDNA Complexes^a

[recA] (μM)	[εDNA]/ [recA]	[NTP] ₀ (μM)	<i>h</i> ^b	<i>K</i> _{app} ^b (μM)
1.2 ^c	3	10-46	2.4	20
0.6 ^c	6	19-66	3.3	26
0.6 ^c	12	19-54	4.3	28
0.6 ^c	18	20-48	4.9	27
0.6 ^d	12	20-49	2.5	33
0.8 ^e	27	14-50	3.3	18
1.2 ^f	3	0.3-1.2	3.3-4.2	0.4-0.6
0.5 ^f	18	0.2-1.1	3.3-3.4	0.4-0.5

^a Performed under standard conditions as described in the text. ^b *h* is the slope of the Hill equation plot (or its kinetic equivalent), and *K*_{app} is the [NTP] required for half-saturation. ^c In these ATP binding experiments, no correction to [ATP]₀ was made for the minor hydrolysis that occurred during the time required for the measured fluorescence to reach its maximum. The average value for *K*_{app} is 25 ± 2 μM. ^d This ATPase run with εDNA gave *V*_m = 3.7 ± 0.1 M min⁻¹ for 480-750 μM ATP, so *k*_{cat} = 6.3 min⁻¹. The Hill plot consisted of six points, encompassed a range for *V*/*V*_m of 0.2-0.75, and showed a correlation coefficient of 0.991. ^e For this φX174 ssDNA promoted ATPase at pH 8.1, 30 °C, *k*_{cat} = 7 min⁻¹ (Weinstock et al., 1981b). ^f Analysis of these ATPγS binding experiments is described in the text. We have indicated the range of possible values for *h* and *K*_{app}.

Table II demonstrates that the four derived values for *K*_{app}, the value of [ATP] required for Δ*F* = Δ*F*_m/2, are in good agreement. The magnitude of the Hill coefficient establishes that ATP converts recA-εDNA into ATP-recA-εDNA in a highly cooperative process under all conditions examined. The degree of cooperativity rises as the ratio [εDNA]/[recA] is increased, but the difference between the *h* values for the two runs at excess εDNA is barely significant. It is not experimentally feasible to increase the [εDNA] further.

The recA-εDNA complex is an effective ATPase under conditions nearly identical with those employed for one set of binding runs. The two experiments afford reasonably similar values for *h* and *K*_{app} (Table II; comparable ATPase data from

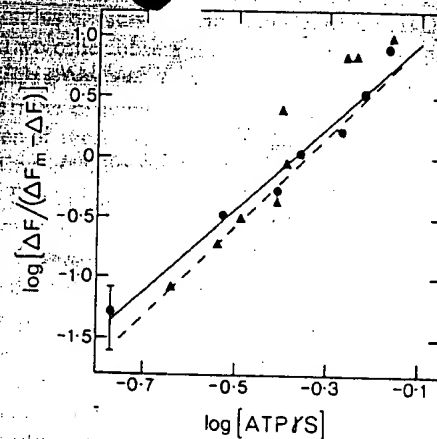


FIGURE 5: Binding of ATPγS to the recA-εDNA complex under standard conditions. The data are plotted according to the Hill equation (eq 1) with [ATPγS] values expressed in micromolar. The broken line is the least-squares straight line through the lowest five (Δ) points. The experiment employed 1.2 μM recA protein and 3.7 μM εDNA. The solid line is the least-squares straight line through all (●) points. That experiment involved 0.5 μM recA protein and 8.9 μM εDNA. The analysis, explained in more detail in the text, assumed that ATPγS binds to the recA-εDNA complex with a stoichiometry of 1:1.

the literature are also shown).

The method described for evaluating the binding of ATP to the recA-εDNA complex has also been applied to ATPγS. Complexes between ATPγS and recA-ssDNA have figured prominently in previous work with recA protein, primarily because they are readily captured in filter-binding assays (Weinstock et al., 1981c). The high stability of the ATPγS-recA-εDNA complex has proven a liability in the fluorescence investigations of ATPγS binding. Analysis of the experimental data requires making allowance for the substantial fraction of the added ATPγS that is bound to recA protein. Furthermore, experiments with excess recA protein witness a reproducible discontinuity in fluorescence at [ATPγS] ≈ 0.8 μM [Figure 5 at log [ATPγS] = -0.4; the point shown at log [Δ*F*/(Δ*F*_m - Δ*F*)] = 0.39 represents three identical determinations] when the solution slowly grows perceptibly hazy. This is one of two instances where enzyme aggregation has caused a problem. It apparently does not affect the value for Δ*F*_m. The measured Δ*F*_m for the hazy solution is identical with that for a clear solution obtained by saturating the pure recA-εDNA complex with a single addition of ATPγS. As a result of these difficulties, the ATPγS binding data are not as reliable as those for ATP. Table II lists the range of values for *h* and *K*_{app} that is compatible with our experiments and Figure 5 displays the Hill equation plots. The entries in Table II summarize several alternative analyses of the data acquired. We can assert confidently that ATPγS converts recA-εDNA to ATPγS-recA-εDNA in a highly cooperative process that shows a *K*_{app} ≈ 0.5 μM. This value of *K*_{app} agrees with the value of *K*_i = 0.6 μM obtained from ATPγS inhibition of the ATPase reaction (Weinstock et al., 1981c).

The tight binding of ATPγS to the recA-εDNA complex presents the opportunity of determining the stoichiometry of that binding by fluorescence titration. Meeting the requirement that [recA-εDNA] greatly exceed the dissociation constant for the ATPγS complex proved difficult. Enzyme aggregation foiled our efforts to use 17 μM recA protein ([εDNA]/[recA] = 7.6). Solutions of 4.92 μM recA protein ([εDNA]/[recA] = 13.7) appeared to remain homogeneous. Triplicate determinations give [ATPγS]/[recA-εDNA] = 0.78 ± 0.04. Various other procedures have given values of 0.5-1.7 for this stoichiometry (Weinstock et al., 1981c).

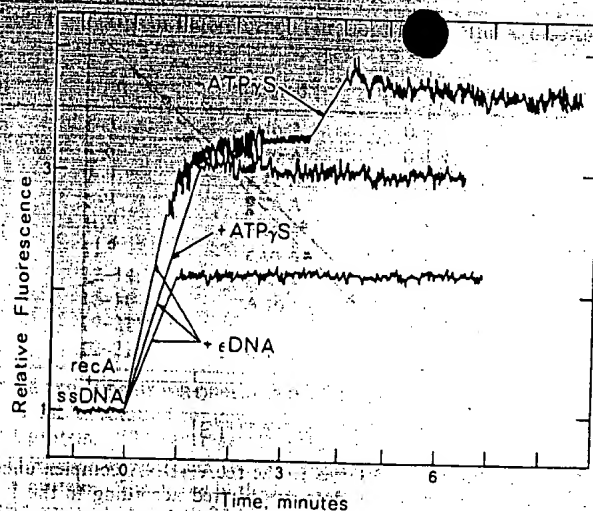


FIGURE 6: Demonstration of the transfer of *recA* protein from ssDNA to ϵ DNA. The extreme left records the fluorescence of a solution containing $0.6 \mu\text{M}$ *recA* protein plus $80 \mu\text{M}$ ssDNA. The lowest run also held $30 \mu\text{M}$ ATP γ S. At the designated point, [ϵ DNA] was increased to $3.6 \mu\text{M}$, and, after ~ 10 s (middle trace) or 3 min (upper trace), [ATP γ S] was raised to $30 \mu\text{M}$. A fast pen response was initially used in the latter in order to demonstrate the occurrence of a detectable time-dependent rise in fluorescence.

Detection of the Transfer of *recA* Protein between Polynucleotides. If ϵ DNA is added to *recA* protein or vice versa, the measured fluorescence reaches its final value by the time observation begins, ~ 20 s after mixing. That is not the case if ϵ DNA is added to a solution containing *recA* protein plus ssDNA. When $3.7 \mu\text{M}$ ϵ DNA is added to a mixture of $0.6 \mu\text{M}$ *recA* with $80 \mu\text{M}$ ssDNA, the last 20–25% of a rapid rise in fluorescence is seen (note the upper curve of Figure 6). The ultimate value, attained within 2 min of mixing, is identical with that found when the same concentrations of *recA* protein and ϵ DNA are combined. Addition of $80 \mu\text{M}$ ssDNA to the latter solution fails to affect its fluorescence significantly.

A large number of such experiments have been performed. We shall focus on typical observations that best illuminate the properties of the various *recA*– ϵ DNA complexes with which we have been concerned or that mostly clearly indicate the potential utility of fluorescent ϵ DNA for investigating protein–nucleic acid interactions. We are currently attempting to acquire more thermodynamic and kinetic data for some of the processes described with the aid of conventional and stopped-flow fluorimeters.

Only one interpretation of the fluorescence change seen when ϵ DNA is added to the solution of the *recA*–ssDNA complex warrants consideration. We are detecting the relatively slow transfer of *recA* protein from ssDNA to ϵ DNA, possibly by a dissociation mechanism. The rate of transfer is too fast to measure accurately with the Perkin-Elmer fluorometer. Since under the specified conditions transfer is more than half complete by the time recording commences, the half-life for the process is considerably less than 30 s. The corresponding value for k , $>1.4 \text{ min}^{-1}$ (Table III), represents a conservative lower limit. The ability of $3.7 \mu\text{M}$ ϵ DNA to strip *recA* protein from $80 \mu\text{M}$ ssDNA so effectively proves, moreover, that *recA* protein preferentially binds to the former polynucleotide.

A simple experiment, illustrated in Figure 6, confirms that *recA* protein transfers from ssDNA to ϵ DNA in the experiment described. It relies upon the fact that a high concentration of ATP γ S “freezes” *recA* protein to the polynucleotide upon which it resides for the few minutes required to record a stable fluorescence intensity (Weinstock et al., 1981c, and

Table III: Apparent First-Order Rate Constants Governing the Transfer of *recA* Protein from a Polynucleotide to Another

Reaction	NTP	k^c (min^{-1})
<i>recA</i> – ϵ DNA + poly(dT) ^a	none	$>6^d$
	ATP	0.13
<i>recA</i> –ssDNA + ϵ DNA ^b	ATP γ S	<0.002
	none	$>1.4^d$
	ATP	0.3–1
	ATP γ S	<0.007

^a These three experiments employed $0.6 \mu\text{M}$ *recA* protein, $3.7 \mu\text{M}$ ϵ DNA, $25 \mu\text{M}$ poly(dT), and, where appropriate, $770 \mu\text{M}$ ATP or $60 \mu\text{M}$ ATP γ S. ^b These three experiments utilized $0.6 \mu\text{M}$ *recA* protein, $80 \mu\text{M}$ ssDNA, $3.7 \mu\text{M}$ ϵ DNA, and, where appropriate, $960 \mu\text{M}$ ATP or $25 \mu\text{M}$ ATP γ S. ^c The text explains the origins of the tabulated values of k . ^d Preliminary results with the stopped-flow fluorometer indicate these rate constants exceed 20 min^{-1} and that the kinetic processes governing these transfers are reasonably complicated.

below). The crucial experiment is performed by adding ϵ DNA to a solution of *recA*–ssDNA in the cuvette, inverting the cuvette twice, adding ATP γ S ~ 10 s after the addition of ϵ DNA, mixing, and recording the final fluorescence intensity. The measured value of 518 exceeds that obtained (365) when ATP γ S is introduced prior to the addition of ϵ DNA, which fixes *recA* protein to ssDNA. It lies below that measured (650) if ATP γ S addition is delayed until 3 min after the addition of ϵ DNA, when transfer of *recA* protein to ϵ DNA is complete. When the addition of ATP γ S occurred ~ 25 s after that of ϵ DNA, the final fluorescence intensity was 605 (not shown in Figure 6).

The fluorescence experiments confirm the extreme inertness of the ATP γ S–*recA*–ssDNA complex (Weinstock et al., 1981c). Note, for example, the relatively constant fluorescence intensity characterizing the lowest curve in Figure 6. The rate of transfer of *recA* protein to ϵ DNA from this complex is too slow to be evaluated accurately by the fluorescence technique. We have estimated the rate as follows. When *recA* protein, ssDNA, and ATP γ S (0.6 , 80 , and $25 \mu\text{M}$, respectively) are mixed and $3.7 \mu\text{M}$ ϵ DNA is added, the initial instantaneous rise in fluorescence associated with the introduction of ϵ DNA is followed by a very slow subsequent rise. Let us assume that the latter corresponds to the formation of ATP γ S–*recA*– ϵ DNA and that eventually all the *recA* protein would be converted to that complex under these conditions. The measured increase in fluorescence after 46 min corresponds to $\sim 25\%$ of the total expected rise. The half-life for the transfer is certainly ≥ 100 min, corresponding to $k \leq 0.007 \text{ min}^{-1}$ (Table III). Weinstock et al. (1981c) report a comparable half-life for the exchange of ATP γ S in the ATP γ S–*recA*–ssDNA complex at 37°C and pH 7.5.

recA protein complexes incorporating ATP are of the most biochemical interest. Consider what happens in the experiment just described when $960 \mu\text{M}$ ATP is substituted for ATP γ S. The instantaneous rise in fluorescence attendant upon the addition of ϵ DNA is followed by a further modestly rapid increase. The final fluorescence intensity, reached after 15–20 min, is identical (within experimental error) with that obtained for a solution holding just ATP, *recA* protein, and ϵ DNA at the same concentrations. A first-order plot of the kinetic data is biphasic, corresponding to an initial slope of 1 min^{-1} and a final one of 0.3 min^{-1} (Table III). There are two important points: (1) the ATP–*recA*–ssDNA complex transfers *recA* protein more rapidly than does the corresponding ATP γ S complex but less rapidly than does *recA*–ssDNA; and (2) *recA* protein binds more strongly to ϵ DNA than to ssDNA, in the presence of ATP.

Complementary experiments have been performed in which recA protein distributes itself between ϵ DNA and poly(dT). Since the affinity of recA protein for poly(dT) is so great (McEntee et al., 1981a), these experiments have been mostly performed in the following way. The desired recA- ϵ DNA complex is prepared; a high concentration of poly(dT) is added, and the fall in fluorescence characterizing the transfer of recA protein from ϵ DNA to poly(dT) is recorded. Unlike the experiments involving the transfer of recA protein from ssDNA to ϵ DNA, it is here possible to assume that the concentration of the polynucleotide acceptor, poly(dT), remains constant during a run. If $1.5\text{--}3.6\text{ }\mu\text{M}$ poly(dT) is added to a mixture of $0.3\text{ }\mu\text{M}$ recA protein with $0.9\text{ }\mu\text{M}$ ϵ DNA, we see the very end of a time-dependent fall in fluorescence. However, under the standard conditions specified in Table III with $[\text{poly(dT)}]_0 = 29\text{ }\mu\text{M}$, transfer is complete within 20 s (the half-life is certainly $< 7\text{ s}$, corresponding to $k > 8\text{ min}^{-1}$). If $25\text{ }\mu\text{M}$ ATP γ S is added before the poly(dT), under the same conditions $< 8\%$ of the expected fall in fluorescence occurs in 37 min ($k < 0.002\text{ min}^{-1}$). Most interesting is the transfer of recA protein in the presence of high [ATP]. Under standard conditions, the reaction obeyed the first-order rate law to better than three half-lives, $k = 0.13\text{ min}^{-1}$. Indeed, five runs, incorporating a range of concentrations, gave excellent first-order plots and identical rate constants [average $k = 0.13 \pm 0.01\text{ min}^{-1}$ for $0.6\text{--}1.8\text{ }\mu\text{M}$ recA protein, $3.7\text{--}40\text{ }\mu\text{M}$ ϵ DNA, and $12\text{--}29\text{ }\mu\text{M}$ poly(dT)]. When the recA- ϵ DNA complex is mixed with a saturating concentration of ATP in the absence of poly(dT), the fall in fluorescence is negligible during the period required for these kinetic measurements (note the final traces in Figure 3).

Discussion

The assumption that the fluorescence data described under Results reveal the existence of recA- ϵ DNA, ATP γ S-recA- ϵ DNA, and ATP-recA- ϵ DNA complexes renders those data readily understandable. It provides a theoretical interpretation that is consistent with previous studies on the interactions of recA protein with ssDNA. Filter-binding assays have captured recA-ssDNA and ATP γ S-recA-ssDNA complexes, while an ATP-recA-ssDNA complex must be implicated in the ssDNA-promoted ATPase activity of recA protein (McEntee et al., 1981a; Weinstock et al., 1981a). Additional telling fluorescence observations are the following: (1) complexes between recA protein and poly(ϵ A) are not detectable (controls in Figures 1 and 2); (2) if Ca^{2+} is substituted for Mg^{2+} or if no divalent cation is present in the standard buffer, the fluorescence enhancement attributed to the conversion of the recA- ϵ DNA complex into the ATP-recA- ϵ DNA ones is not seen; (3) addition of ADP to a solution of the recA- ϵ DNA complex causes the fluorescence intensity to fall slightly; and (4) the fluorescence data imply that the recA-ssDNA and recA- ϵ DNA complexes rapidly transfer recA protein to suitable acceptor polynucleotides while for the corresponding ATP γ S complexes those transfers are extremely slow. All four points find close analogy in earlier work, employing other techniques (Cotterill et al., 1982; McEntee et al., 1981a; Weinstock et al., 1981a): (1) recA protein binds poorly to poly(rA); (2) filter-binding assays fail to detect an ATP γ S-recA-ssDNA complex in the absence of a divalent cation (Ca^{2+} affords that complex in reduced yield but does not support ATPase activity); (3) ADP appears to promote the dissociation of recA protein from ssDNA; and (4) filter-binding assays establish that the recA-ssDNA complex is highly mobile while the corresponding ATP γ S one is extremely inert. These several arguments justify the conclusion that the

interactions of recA protein with ϵ DNA and ssDNA are similar. Two other observations corroborate the point: ϵ DNA and ssDNA support the ATPase activity of recA protein with comparable efficiency (Table II) and the relative ease of transfer of recA protein from the three kinds of complexes identified is comparable for the two polynucleotides (Table III). The enhanced affinity of ϵ DNA for recA protein is experimentally advantageous. We believe it derives, at least in part, from the disrupted secondary structure of ϵ DNA. The etheno derivatives that characterize ϵ DNA cannot participate in base pairing.

In brief, fluorescence studies with ϵ DNA should provide biochemically relevant insights into the behavior of recA protein and other proteins that interact strongly with ssDNA. The experiments performed thus far with recA protein (1) define the stoichiometry governing its binding to ϵ DNA under various conditions, (2) quantify the binding of ATP to the recA- ϵ DNA complex, (3) quantify the binding and stoichiometry of the binding of ATP γ S to the recA- ϵ DNA complex, and (4) provide initial estimates on the rate at which recA protein transfers between polynucleotides. Our discussion will focus on the titration experiments and on the data bearing on the properties of the ATP-recA- ϵ DNA complex.

Stoichiometry for the Binding of recA Protein to ϵ DNA. The fluorescence titration procedures described offer a convenient empirical method for standardizing recA protein solutions. The experiments may be performed rapidly (15–20 min per run) and yield highly reproducible equivalence points, which are readily estimated by eye to $\pm 5\%$ under the specified conditions. Table I summarizes our results and displays titration data for two other batches of recA protein (runs 10 and 11).

Earlier experiments of various kinds have indicated that each recA protein monomer binds to 4–10 nucleotides of ssDNA [e.g., see Craig & Roberts (1980) and McEntee et al. (1981b)]. Our determinations all lie within that range. Nevertheless, the order of addition of the reagents appears to affect the titration results significantly, and it is instructive to examine why this may be so. The stoichiometry values obtained by adding ϵ DNA to recA protein are more readily interpreted. The procedure maximizes the likelihood that recA protein fully coats ϵ DNA throughout the titration, since it is in excess until the equivalence point. The good agreement among the results from runs 1–9 confirms this expectation. The conclusion that each recA monomer covers 6.0 ± 0.3 nucleotides of ϵ DNA probably represents the best available estimate for the stoichiometry of binding between the protein and single-stranded polynucleotides.

Titration 12–21, where recA protein is added to ϵ DNA, give larger values for the ratio $[\epsilon\text{DNA}]/[\text{recA}]$ at the equivalence point. We think these experiments overestimate the ability of recA protein to cover ϵ DNA. The fluorescent regions of ϵ DNA cannot be homogeneously distributed, since the adenosine residues are not so distributed and the modification reaction with chloroacetaldehyde may not act randomly [cf. Ledneva et al. (1978)]. The finding that recA protein binds far more strongly to ϵ DNA than it does to ssDNA suggests that recA protein may bind preferentially to the modified regions of the ϵ DNA strands. Should this be so, when ϵ DNA is in excess, recA protein will afford a fluorescence enhancement that overestimates the total degree of coverage of ϵ DNA. This describes exactly the situation that pertains at the early stages of titration 12–21, when recA protein is added to ϵ DNA.

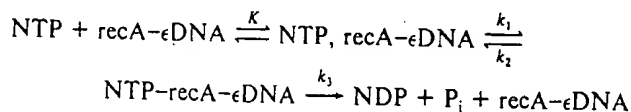
The fact that the extent of overestimation is greatest for runs 17–21, which includes ATP γ S, accords with the preceding

explanation. Recall that recA protein binds essentially irreversibly to ϵ DNA in the presence of ATP γ S. Runs 17–21 should therefore most clearly reveal the initial preferred attachment of recA protein to the modified regions of ϵ DNA, and they do. The high mobility of recA protein in the simple recA- ϵ DNA complex affords the protein ample opportunity to reposition itself on the ϵ DNA strands during the course of titrations 12–14. This repositioning reduces the extent to which those runs overestimate the ability of recA protein to cover ϵ DNA. Perhaps fortuitously, the ATP-recA- ϵ DNA complex exhibits both intermediate mobility and an intermediate degree of overestimation in runs 15–16.

ATP-recA- ϵ DNA Complex. ATP converts recA- ϵ DNA into ATP-recA- ϵ DNA in a highly cooperative process under all conditions examined (Table II). The values for h and K_{app} obtained from the Hill equation are very close to those obtained for the ϵ DNA- and ssDNA-stimulated ATPase reactions. It is gratifying that such different techniques are in good agreement, for the critical fluorescence observations are completed when the ATPase reaction has barely commenced. This agreement reinforces our conviction that we are justified in ascribing the fluorescence change seen, when ATP is added to the recA- ϵ DNA complex, to the formation of an ATP-recA- ϵ DNA complex.

We do not yet know what that fluorescence change represents, at a molecular level. Preliminary studies establish the feasibility of examining the binding of ATP to the recA- ϵ DNA complex with a stopped-flow fluorometer. They indicate that it will not be easy to establish a quantitative link between the fluorescence and ATPase data. The measured fluorescence intensity in the ATP binding runs clearly does not depend solely upon the instantaneous ATP concentration. The fluorescence intensity noticeably rises initially (Figure 3), but ATP is converted to ADP without a detectable lag (Weinstock et al., 1981a). Qualitatively, the fall in fluorescence seen after the peak value is reached (Figure 3) coincides with falling [ATP]. Quantitatively, the ATPase rate estimated fluorometrically (via the appropriate Hill plot) consistently exceeds that measured conventionally by at least 2-fold.

What do the fluorescence experiments contribute to the question of the role of ATP in the recA protein system? It is helpful to start by contrasting the behavior of ATP and ATP γ S. Both readily form ternary ssDNA complexes, but ATP is cleaved some 5000 times more rapidly (Craig & Roberts, 1981) and apparently binds to recA- ϵ DNA \sim 50 times less tightly. The following highly schematic equation (where NTP-recA- ϵ DNA symbolizes the complex detected in the fluorometer and responsible for the cleavage reactions) suggests one possible way to reconcile these facts:



For ATP γ S, $k_2 \gg k_3$ and binding experiments afford a true equilibrium dissociation constant, $K(k_2/k_1)$. For ATP, $k_3 \gg k_2$. The k_3 route offers a relatively rapid pathway for breakdown of the critical ATP complex. ATP binding experiments thus measure an apparent dissociation constant for ATP, $K(k_3/k_1)$, that significantly exceeds its true dissociation

constant from ATP-recA- ϵ DNA.

The data in Table III provide the first firm evidence that the ATP-recA- ϵ DNA and ATP-recA-ssDNA complexes release recA protein far more rapidly than do the corresponding complexes with ATP γ S (perhaps the ATP complexes have escaped detection in filter-binding experiments because of this lability). It is thus tempting to link that release to the cleavage of ATP in the k_3 step of the above equation. However, if the two processes are coupled, the coupling is quite inefficient under our experimental conditions. The rate constant of 0.13 min $^{-1}$ for the transfer of recA protein from ϵ DNA to poly(dT) in the presence of ATP is far smaller than the ATPase turnover number of 6.3 min $^{-1}$. The discrepancy with unmodified ssDNA is less but still substantial. The primary function of the ATPase activity may well lie elsewhere (Cox & Lehman, 1981; Weinstock et al., 1981a).

Acknowledgments

A sabbatical leave from Amherst College to M.S.S. enabled him to participate in this project.

References

- Ames, B. N. (1966) *Methods Enzymol.* 8, 115–118.
- Cotterill, S. M., Satterthwait, A. C., & Fersht, A. R. (1982) *Biochemistry* 21, 4332–4337.
- Cox, M. M., & Lehman, I. R. (1981) *Proc. Natl. Acad. Sci. U.S.A.* 78, 3433–3437.
- Craig, N. L., & Roberts, J. W. (1980) *Nature (London)* 283, 26–30.
- Craig, N. L., & Roberts, J. W. (1981) *J. Biol. Chem.* 256, 8039–8044.
- DasGupta, C., & Radding, C. M. (1982) *Proc. Natl. Acad. Sci. U.S.A.* 79, 762–766.
- Geider, K., & Hoffmann-Berling, H. (1981) *Annu. Rev. Biochem.* 50, 233–260.
- Krzyzusia, W. J., Biernat, J., Ciesiolka, J., Gulewicz, K., & Wiewiorowski, M. (1981) *Nucleic Acids Res.* 9, 2841–2851.
- Ledneva, R. K., Razjivin, A. P., Kost, A. A., & Bogdanov, A. A. (1978) *Nucleic Acids Res.* 5, 4225–4243.
- Lee, C. H., & Wetmur, J. G. (1973) *Biochem. Biophys. Res. Commun.* 50, 879–885.
- Leonard, N. J., & Tolman, G. L. (1975) *Ann. N.Y. Acad. Sci.* 255, 43–58.
- McEntee, K., Weinstock, G. M., & Lehman, I. R. (1981a) *J. Biol. Chem.* 256, 8835–8844.
- McEntee, K., Weinstock, C. M., & Lehman, I. R. (1981b) *Prog. Nucleic Acid Res. Mol. Biol.* 26, 265–281.
- Radding, C. M. (1981) *Cell (Cambridge, Mass.)* 25, 3–4.
- Steiner, R. F., Kinnier, W., Lunasin, A., & Delac, J. (1973) *Biochim. Biophys. Acta* 294, 24–37.
- Toulm , J. J., & H l ne, C. (1980) *Biochim. Biophys. Acta* 606, 95–104.
- Weinstock, G. M., McEntee, K., & Lehman, I. R. (1981a) *J. Biol. Chem.* 256, 8829–8834.
- Weinstock, G. M., McEntee, K., & Lehman, I. R. (1981b) *J. Biol. Chem.* 256, 8845–8849.
- Weinstock, G. M., McEntee, K., & Lehman, I. R. (1981c) *J. Biol. Chem.* 256, 8850–8855.
- West, S. C., Cassuto, E., & Howard-Flanders, P. (1981) *Proc. Natl. Acad. Sci. U.S.A.* 78, 6149–6153.

- Hartmann, K.-U., and Heidelberger, C. (1961), *J. Biol. Chem.* 236, 3006.
- Heidelberger, C. (1970), *Cancer Res.* 30, 1549.
- Heidelberger, C., and Anderson, S. W. (1964), *Cancer Res.* 24, 1969.
- Heidelberger, C., Chaudhuri, N. K., Danneberg, P., Mooren, D., Griesbach, L., Duschinsky, R., Schnitzer, R. J., Plevin, E., and Schneier, J. (1957), *Nature (London)* 179, 663.
- Heidelberger, C., Griesbach, L., Montag, B. J., Mooren, D., Cruz, O., Schnitzer, R. J., and Grunberg, E. (1958), *Cancer Res.* 18, 305.
- Heidelberger, C., Kaldor, G., Mukherjee, K. L., and Danneberg, P. B. (1960), *Cancer Res.* 20, 903.
- Heidelberger, C., Parsons, D. G., and Remy, D. G. (1964), *J. Med. Chem.* 7, 1.
- Heller, S. R. (1968), *Biochem. Biophys. Res. Commun.* 32, 998.
- Kalman, T. I. (1971), *Biochemistry* 10, 2567.
- Kalman, T. I., and Bardos, T. J. (1970), *Mol. Pharmacol.* 6, 621.
- Kalman, T. I., and Fung, S.-M. (1972), 164th National Meeting of the American Chemical Society, New York, N. Y., Sept, Abstract BIOL.
- Laemmli, U. K. (1970), *Nature (London)* 227, 680.
- Langenbach, R. J., Danenberg, P. V., Fridland, A., Cleland, W. W., and Heidelberger, C. (1972a), *Fed. Proc., Fed. Amer. Soc. Exp. Biol.* 31, 419Abstr.

- Langenbach, R. J., Danenberg, P. V., and Heidelberger, C. (1972b), *Biochem. Biophys. Res. Commun.* 48, 1565.
- Lomax, M. I. S., and Greenberg, G. R. (1967), *J. Biol. Chem.* 242, 1302.
- Pastore, E. J., and Friedkin, M. (1962), *J. Biol. Chem.* 237, 3802.
- Reyes, P., and Heidelberger, C. (1965), *Mol. Pharmacol.* 1, 14.
- Santi, D. V., and Brewer, C. F. (1968), *J. Amer. Chem. Soc.* 90, 6236.
- Santi, D. V., Brewer, C. F., and Farber, D. (1970), *J. Heterocycl. Chem.* 7, 903.
- Santi, D. V., and McHenry, C. S. (1972), *Proc. Nat. Acad. Sci. U. S.* 69, 1855.
- Santi, D. V., and Sakai, T. T. (1971), *Biochemistry* 10, 3598.
- Sharma, R. K., and Kisliuk, R. L. (1973), *Fed. Proc., Fed. Amer. Soc. Exp. Biol.* 32, 591Abstr.
- Slavik, K., and Zakrzewski, S. F., (1967), *Mol. Pharmacol.* 3, 370.
- Wellings, P. C., Audry, P. N., Borg, F. H., Jones, B. R., Broom, D. C., and Kaufman, H. E. (1972), *Amer. J. Ophthalmol.* 73, 932.
- Yoshikawa, M., Kato, T., and Takenishi, T. (1967), *Tetrahedron Lett.*, 5065.

Guanosine Triphosphate and Guanosine Diphosphate as Conformation-Determining Molecules. Differential Interaction of a Fluorescent Probe with the Guanosine Nucleotide Complexes of Bacterial Elongation Factor Tu†

Laura J. Crane* and David Lee Miller

ABSTRACT: Tritium exchange studies have recently provided evidence that conformational differences between EFTu-GTP and EFTu-GDP may account for the differential binding of AA-tRNA by EFTu-GTP (Printz, M. P., and Miller, D. L. (1973), *Biochem. Biophys. Res. Commun.* 53, 149). These conformational differences have been further characterized by studying the interaction of the fluorescent dye 1-anilino-8-naphthalenesulfonate with EFTu-GTP and EFTu-GDP. EFTu-GTP enhances the fluorescence of 1-anilino-8-naphthalenesulfonate to a greater extent than does EFTu-GDP. When EFTu-GTP is complexed with Phe-tRNA, however, its interaction with 1-anilino-8-naphthalenesulfonate increases the fluorescence of the dye only as much as EFTu-GDP does. Titration of a solution of the dye with excess protein shows that both EFTu-GTP and EFTu-GDP produce the same fluorescence enhancement, about 200-fold, for the tightest bound dye. Equilibrium dialysis binding measurements indi-

cate that EFTu-GTP binds three molecules of the sulfonate dye with an apparent $K_{diss} \approx 2 \times 10^{-5}$ M, whereas EFTu-GDP binds two molecules with an apparent $K_{diss} \approx 5-8 \times 10^{-5}$ M. Both complexes have at least one other population of more weakly bound dyes. It would appear from these data that differences in conformation between EFTu-GTP and EFTu-GDP are centered chiefly in a region of EFTu-GTP sensitive to AA-tRNA binding. However, further analysis of the fluorescence data indicates that somewhat more extensive conformational differences exist between the two nucleotide complexes of EFTu. Slope changes in the curve of the titration of 1-anilino-8-naphthalenesulfonate by EFTu-GTP and in Scatchard plots of the fluorescence data indicate cooperativity in the fluorescence yield and thus interaction of the dye binding sites on EFTu-GTP. EFTu-GDP gives no evidence of site interaction.

Nucleoside triphosphates perform three distinct functions in organisms. They may be reagents or intermediates in

the synthesis or degradation of cellular components, where the formation of a phosphate ester intermediate is a favorable pathway for removing or adding the elements of water. The synthesis and degradation of glycogen are examples of this function. In contrast to these reactions, nucleoside triphos-

† From the Roche Institute of Molecular Biology, Nutley, New Jersey 07110. Received August 17, 1973.

phates also promote cellular processes where phosphate ester formation seems to play no obligatory role. Among the examples of this function are motile and contractile processes. A third and possibly related function of these compounds is to control biochemical reactions, as CTP regulates the activity of aspartate transcarbamoylase.

The role of GTP in the binding of aminoacyl-tRNA (AA-tRNA)¹ to ribosomes bears a resemblance both to the function of ATP in motile systems and to the regulatory role of CTP. In the process of protein biosynthesis in prokaryotes, GTP promotes the binding of AA-tRNA to ribosomes in the presence of mRNA and elongation factor Tu (EFTu).² Aminoacyl-tRNA and EFTu-GTP readily form the ternary complex AA-tRNA-EFTu-GTP (Weissbach *et al.*, 1970), which interacts with the mRNA-ribosome complex. As a result of this interaction, AA-tRNA is bound to the ribosome, the GTP in the ternary complex is hydrolyzed, and EFTu-GDP, which does not bind to AA-tRNA, is released.

The EFTu-GDP complex is very stable, having a dissociation constant in the range of 10^{-8} – 10^{-9} M, whereas EFTu-GTP is about 100-fold less stable. The EFTu-GDP complex dissociates very slowly by itself; however, another protein, EFTs, catalyzes the exchange of GTP for GDP, thus completing the cycle of reactions in the binding process.

The details of this function of EFTu remain undetermined. There is some evidence that it alters the structure of the ribosome (Chuang and Simpson, 1971). Other possibilities, at present unproved, are that the protein alters the structure of AA-tRNA, or provides additional binding sites for interaction of the ternary complex with the ribosome. Whatever the function of EFTu in peptide chain elongation, the specificity of its interactions seems to be determined by which guanosine nucleotide is bound to it. Thus, the dissociation constant for AA-tRNA from EFTu-GTP is 10^{-8} M or less (Miller *et al.*, 1973), whereas EFTu-GDP does not interact with AA-tRNA to a measurable extent; the dissociation constant of the hypothetical AA-tRNA-Tu-GDP complex must be greater than 10^{-4} M.

We have postulated that this difference in reactivity is due to conformational differences between EFTu-GTP and EFTu-GDP. A previous study of tritium exchange rates provided evidence to support this concept (Printz and Miller, 1973). Tritiated EFTu-GTP exchanged some of its peptide bond hydrogens considerably more rapidly than EFTu-GDP did, and at certain times in the exchange process EFTu-GDP possessed about 50% more unexchanged hydrogens than EFTu-GTP, suggesting that GDP induces a tightening of at least a portion of the tertiary structure of EFTu. In an effort to localize the conformational differences between the two complexes, and to relate them to their differential interaction with AA-tRNA, we have examined the binding of a fluorescent dye, 1-anilino-8-naphthalenesulfonate, to the complexes of EFTu. Although this molecule has been shown to interact with a large number of proteins, the number of fluorescent binding sites per protein is usually small (Stryer, 1965; Daniel and Weber, 1966; Brand, 1970). Furthermore, the fluorescence yield of the resulting protein-dye complex depends markedly upon the properties of the dye binding site, and is thought to increase with the hydrophobicity of the binding site (Brand and Gohlke, 1972).

Materials and Methods

Preparation of EFTu Complexes. GDP, GTP, and dithiothreitol were obtained from Calbiochem. Methylene diphosphonic acid was a product of Miles Laboratories. GMP-PCP was prepared by the morpholidate method (Moffatt and Khorana, 1961) and was crystallized as the disodium salt. The disodium salt of 1-anilino-8-naphthalenesulfonate, obtained from K & K Laboratories, was converted to the magnesium salt, treated with Norite, and recrystallized from water (Stryer, 1965). The molar extinction coefficient of the purified material was found to be 4.9×10^4 at 350 nm. Thin-layer chromatography revealed a single fluorescent component. Purified Phe-tRNA from *Escherichia coli* (1600 pmol/ A_{260}) was prepared from a tRNA mixture (Schwarz-Mann) by benzoylated DEAE-cellulose chromatography (Gillam and Tener, 1971). The preparation of homogeneous EFTu as the EFTu-GDP complex has been described elsewhere (Miller and Weissbach, 1970, 1973). The EFTu-GMP-PCP complex was prepared by passing EFTu-GDP (10 mg) through a 90 × 15 cm column of Bio-Gel P-4 polyacrylamide gel equilibrated with 50 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 1 mM dithiothreitol, and 1 mM GMP-PCP. One pass through the column removed 80–90% of the GDP, whereas two passes removed 95–97% of the GDP.

To ensure that small differences in the protein preparations did not influence the results, both EFTu-GDP and EFTu-GTP were formed from a common intermediate, EFTu-GMP-PCP, by adding a small excess of the appropriate nucleotide. Since GMP-PCP is relatively loosely bound to EFTu, it is readily displaced by GTP or GDP. The extent of conversion of EFTu-GMP-PCP to EFTu-GTP or EFTu-GDP was determined by a Millipore filter assay using the appropriate tritium-labeled nucleotide.

Fluorescence Measurements. All fluorescence measurements were made at 4° using an Aminco-Bowman spectrophotofluorometer with a ratio attachment. The instrument was routinely standardized with a solution of quinine sulfate (12 ppb) in 0.1 N H₂SO₄. The excitation and emission spectra of all the complexes tested were found to be very similar, and an excitation wavelength of 350 nm and an emission wavelength of 470 nm were used for all titrations (the emission maxima for the complexes were approximately 480 nm, but the lower wavelength was used to minimize fluorescence of the free dye in aqueous solution).

All titrations were performed in 0.5-cm cuvetts in a buffer of 50 mM Tris–10 mM MgCl₂–1 mM dithiothreitol, (pH 7.4). The solution to be titrated (250 μ l) was added to the cuvet, the fluorescence was measured, and then microliter increments of titrant were added. After each addition of titrant the solution was stirred with a polyethylene rod and the fluorescence was remeasured after it arrived at a constant value (in titrations of the protein-nucleotide complexes the final fluorescence was achieved immediately; when Phe-tRNA was being added to quench fluorescence, final readings were taken after approximately 2 min).

Three types of titrations were performed: (1) titrations of one of the EFTu complexes with 1-anilino-8-naphthalenesulfonate (the sulfonate dye) to determine the extent of fluorescence enhancement; for these titrations the complex was present in the buffer solution at an initial concentration of 1.0×10^{-6} M in the presence of a threefold excess of nucleotide; the sulfonate solution used for titration was usually 2×10^{-4} M in sulfonate dye; for titrations in the early region of the curve titrant solution of 2×10^{-4} M sulfonate dye was used; (2)

¹ The following abbreviations are used: AA- (or Phe-) tRNA, aminoacyl- (or phenylalanyl-) tRNA; EFTu, EFTs, and EFG, elongation factors Tu, Ts, and G; GMP-PCP, guanylyl methylene diphosphonate.

² For a review on the process of peptide chain elongation, see Lucas-Lenard and Lipmann (1971).

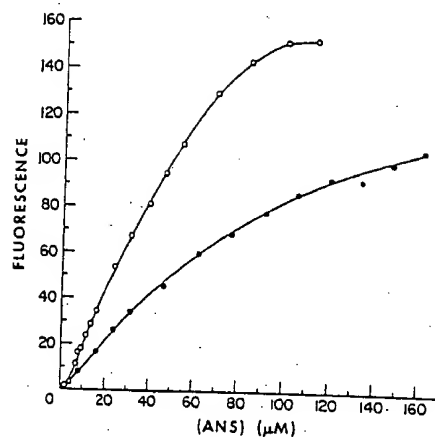


FIGURE 1: Titration of EFTu-GTP and EFTu-GDP by 1-anilino-8-naphthalenesulfonate (ANS): (O) EFTu-GTP; (●) EFTu-GDP. Fluorescence is in arbitrary units.

titration of the sulfonate with an EFTu complex to determine the fluorescence yield of the most tightly bound dye and its affinity for the protein; the dye was present in the buffer solution at an initial concentration of 2×10^{-4} M, to which increments of EFTu complex were added from a solution containing 1×10^{-4} M protein and 3×10^{-4} M nucleotide; (3) titration of a dye-EFTu complex solution with Phe-tRNA to measure its quenching effect; a titration in the early region of the curve was performed as described in 1, followed by incremental additions of microliter amounts of a 6×10^{-5} M solution of Phe-tRNA.

Blank corrections were made as follows. In all titrations corrections were made for dilution (which never exceeded 10%) and absorbance, which reached 0.25 at 350 nm at the highest concentration of dye used. In type 1 titrations correction was made for free dye by the method of Thompson and Yielding (1968). In type 2 titrations an additional correction was made for the contribution of the protein-complex solution to the fluorescence. Finally, the contribution of the Phe-tRNA solution to the measured fluorescence was also corrected for in type 3 titrations.

Equilibrium Dialysis. Equilibrium dialysis was performed at 4° in cells manufactured by Technilab Instruments. The usual buffer solution (0.5 ml) containing a range of 1-anilino-8-naphthalenesulfonate concentrations (4×10^{-5} to 1×10^{-3} M) were placed in both chambers of the dialysis cells. A fixed protein-nucleotide complex concentration of 3×10^{-5} – 1.0×10^{-4} M was included in the solution on one side of each cell. After 5 hr, a time at which control experiments showed dialysis to be complete, the optical density at 350 nm of each chamber was measured, and the value was corrected for the protein contribution. The solutions on both sides of the cell contained an amount of GDP or GTP three times the protein concentration.

Results

Fluorescence of Dye-Tu-GDP and Dye-Tu-GTP. The conformational difference between the two complexes first observed by tritium exchange studies was reflected also in their interaction with the sulfonate dye. The titration curves obtained by adding small increments of a 1-anilino-8-naphthalenesulfonate solution to either EFTu-GDP or EFTu-GTP are shown in Figure 1. Upon addition of dye to either complex, the wavelength of maximum emission shifted from approximately 530 nm (dye in aqueous solution) to 480 nm, similar to the dye in ethanol. Although the excitation and

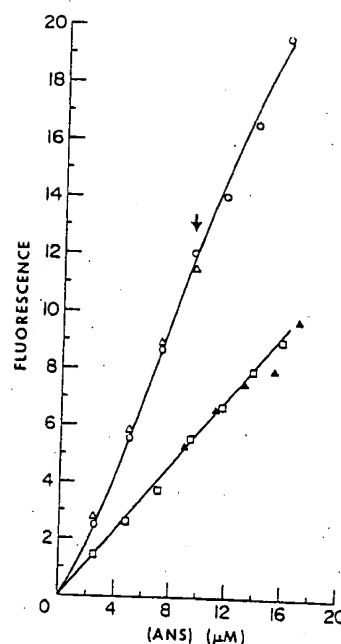


FIGURE 2: Effect of Phe-tRNA upon the fluorescence of dye-EFTu-GTP; comparison with dye-EFTu-GDP. At the point in the titration of EFTu-GTP by 1-anilino-8-naphthalenesulfonate (ANS) marked by an arrow, an equimolar amount of Phe-tRNA was added to the EFTu-GTP solution being titrated: (O) control titration of EFTu-GTP by 1-anilino-8-naphthalenesulfonate (ANS); no Phe-tRNA added during titration; (Δ) titration of EFTu-GTP before addition of Phe-tRNA; (\blacktriangle) titration of EFTu-GTP after addition of Phe-tRNA; (\square) control titration of EFTu-GDP, no Phe-tRNA added during titration.

emission spectra of the two dye-EFTu complexes were identical (data not shown), dye-EFTu-GDP consistently gave considerably less fluorescence than the dye-EFTu-GTP complex throughout the titration.

Effect of Phe-tRNA on the Fluorescence of Dye-EFTu-GTP and Dye-EFTu-GDP. In an attempt to determine whether this difference could be related to specific differences on the surface of EFTu-GTP and EFTu-GDP, we measured the effect of Phe-tRNA on the fluorescence of the protein-dye complexes. Figure 2 shows that a stoichiometric addition of Phe-tRNA to EFTu-GTP midway through sulfonate dye titration caused the fluorescence of the solution to decrease to a value corresponding to an identical concentration of EFTu-GDP. Further increments of dye produced a titration curve similar to the EFTu-GDP titration run as a control. In a parallel experiment, addition of the same amount of Phe-tRNA to EFTu-GDP produced no net change in fluorescence.

The specificity and extent of the fluorescence-diminishing effect of Phe-tRNA was tested by adding increments of Phe-tRNA to a solution of EFTu-GDP or EFTu-GTP pretitrated with 1-anilino-8-naphthalenesulfonate. As Figure 3A shows, a sharp decrease in fluorescence was observed with EFTu-GTP, which leveled off as the Phe-tRNA/(EFTu-GTP) ratio approached unity. As the Phe-tRNA/(EFTu-GTP) ratio was further increased, the fluorescence again decreased, leveling out a second time as the Phe-tRNA/(EFTu-GTP) ratio approached 3. The addition of Phe-tRNA to dye-EFTu-GDP (Figure 3A) caused only a gradual decrease in fluorescence at high concentrations of Phe-tRNA. Deacylated Phe-tRNA had no net effect upon the fluorescence of either dye-protein-nucleotide complex. It would seem that Phe-tRNA has two modes of interaction with EFTu, a specific stoichiometric interaction with the form of EFTu that binds GTP, and a nonspecific interaction that occurs at higher Phe-

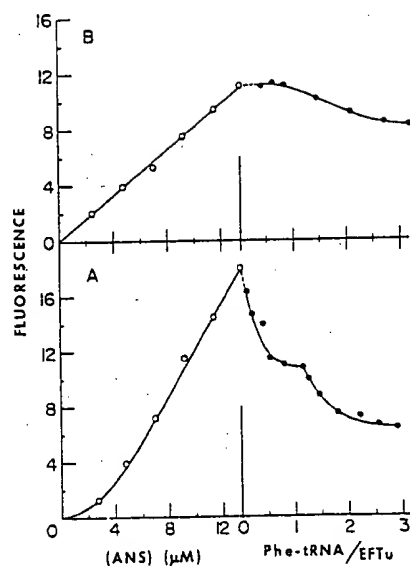


FIGURE 3: Ability of Phe-tRNA to quench fluorescence of dye-EFTu-GDP and dye-EFTu-GTP: (A) EFTu-GTP; (B) EFTu-GDP; (O) titration by 1-anilino-8-naphthalenesulfonate (ANS); (●) fluorescence of titrated solution after incremental additions of Phe-tRNA.

tRNA/EFTu ratios and differentiates much less strongly between the two forms of EFTu.

Quantitation of 1-Anilino-8-naphthalenesulfonate Binding. On the basis of the titration curves, it would appear that sulfonate binding is a sensitive indicator of the conformational differences between the two EFTu complexes that provides for the very selective binding of AA-tRNA by EFTu-GTP. In the absence of further quantitative information, however, it is not possible to conclude whether the conformational difference between the two forms of EFTu is confined to a local area directly involved in AA-tRNA binding, or if a major conformational difference exists with the identity of the EFTu-GDP and AA-tRNA-EFTu-GTP titration curves being a matter of coincidence.

Quantitation of the fluorescence results requires the fluorescence yield(s) of the bound dyes. If all of the bound dyes are approximately equivalent, the fluorescence yield can be obtained by titrating a solution of dye with excess protein (Weber and Young, 1964). Double reciprocal plots of such data are shown for the two forms of EFTu in Figures 4 and

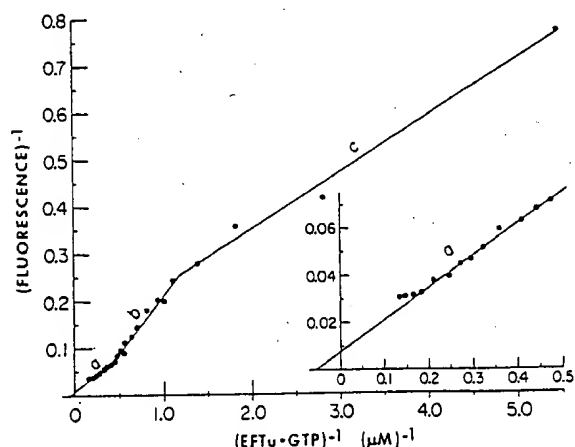


FIGURE 4: Double reciprocal plot of titration of 1-anilino-8-naphthalenesulfonate (ANS) by EFTu-GTP; 0.97×10^{-4} M EFTu-GTP containing 2×10^{-6} M dye was added in small increments to 2×10^{-6} M dye.

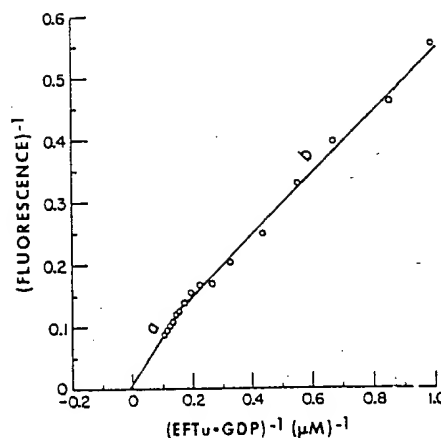


FIGURE 5: Double reciprocal plot of titration of 1-anilino-8-naphthalenesulfonate (ANS) by EFTu-GDP; 0.43×10^{-4} M EFTu-GDP containing 2×10^{-6} M dye was added in small increments to 2×10^{-6} M dye.

5. Both curves show slope changes as the protein concentration increases. In the absence of other information, there are several possible explanations for this type of behavior. The biphasic nature of the EFTu-GDP curve could result from two populations of binding sites having the same fluorescence yield and differing affinities for the dye, or alternatively two populations differing in both characteristics. The EFTu-GTP curve is still more complex, having at least two slope changes in the binding region of interest. Some complexity was expected because of the sigmoidal nature of the early region of the 1-anilino-8-naphthalenesulfonate titration curve (Figure 1). This type of curve usually reflects cooperativity of binding, and the increase in slope in Figure 4 designated as region b is consistent with this interpretation. However, the same type of behavior would be seen if the second population of dye binding sites had a higher fluorescence yield than the first.

Although no simple analysis can be made of regions b and c, in the region of large excess protein (region a) both reciprocal curves extrapolate to the same intercept and give a fluorescence yield for the most tightly bound dye molecule of 70 μ M in arbitrary units, about a 200-fold increase over the fluorescence of 1-anilino-8-naphthalenesulfonate alone in aqueous solution at 470 nm. The apparent dissociation constants determined from the respective reciprocal plots differ, however, extrapolation to the $1/[\text{EFTu}]$ intercept yields $K_{\text{diss}} = 1.7 \times 10^{-5}$ M for EFTu-GTP and 8.0×10^{-5} M for EFTu-GDP. When these fluorescence yield values are used to treat the titration data of the two forms of EFTu by the method of Scatchard *et al.* (1956), the curves shown in Figure 6 result. These curves are subject to the same difficulties in interpretation as the double reciprocal plots. The convergent nature of the curves is to be expected if cooperativity exists among the 1-anilino-8-naphthalenesulfonate binding sites as analyzed and discussed by Cassman and King (1972). However, the same type of curve could result from varying fluorescence yields.

Because of the apparent complexities of the fluorescence binding data, equilibrium dialysis measurements were used to obtain binding information by an independent method, although the amount of EFTu complex required prohibited the extensive use of this technique. The results from these experiments for both forms of EFTu are shown in Figure 7. EFTu-GDP binds a large number of 1-anilino-8-naphthalenesulfonate molecules very weakly. Clearly differentiated from these sites are the two tight-binding sites with an apparent K_{diss}

FIGURE 3: Titration of 1-anilino-8-naphthalenesulfonate (ANS) by EFTu-GDP and EFTu-GTP.

4.7 \times three and tightly!

Inter

func

GDP

EFTu

EFTu

GDP

of the

1-anil

EFTu

the re

divid

no ne

EFTu

soluti

dropp

superi

as we

To th

is an

simila

The

GTP;

cence

GDP,

EFTu

of the

EFTs

GTP

EFTs

Furth

facilit

Discu

Inte

data;

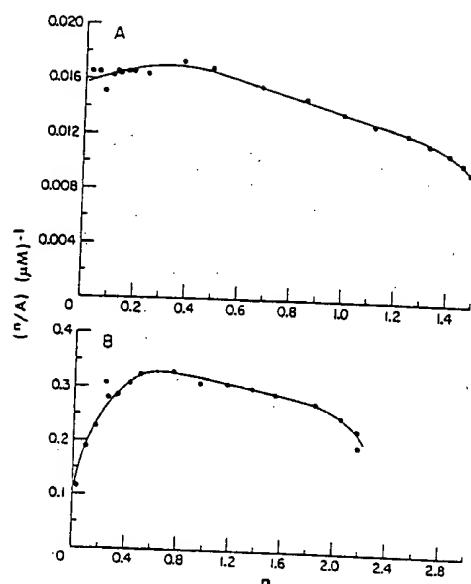


FIGURE 6: Scatchard plots, fluorescence data of 1-anilino-8-naphthalenesulfonate (ANS) titration of EFTu-GTP and EFTu-GDP; n , number of moles of dye bound per mole of EFTu, calculated on the basis of a micromolar fluorescence yield of 70; A, concentration of free dye; (A) EFTu-GDP; (B) EFTu-GTP.

4.7×10^{-5} M. Equilibrium dialysis of dye-EFTu-GTP yields three dye molecules bound tightly ($K_{diss} = 1.9 \times 10^{-5}$ M) and at least two additional molecules bound much less tightly.

Interaction of EFTs with EFTu-GDP and EFTu-GTP. The function of EFTs seems to be to facilitate the replacement of GDP with GTP on EFTu by first displacing GDP, forming EFTu-EFTs; EFTs is then displaced by GTP. *In vitro* the EFTu-EFTs complex can be formed from either EFTu-GDP or EFTu-GTP. It was of interest to determine the nature of the EFTu-EFTs complex in terms of its ability to bind 1-anilino-8-naphthalenesulfonate. When EFTs was added to EFTu-GDP and the solution titrated with dye (Figure 8) the resulting titration curve was equal to the sum of the individual EFTu-GDP and EFTs titration curves, indicating no net effect of EFTs upon the dye-binding properties of EFTu-GDP. When EFTs was added to a partially titrated solution of EFTu-GTP, the net fluorescence of the complex dropped, and further titration produced a curve roughly superimposable upon a control EFTu-GDP titration curve, as would be required by the thermodynamics of the system. To the extent that 1-anilino-8-naphthalenesulfonate binding is an indication of conformation, EFTu in EFTu-EFTs is similar to that form which binds GDP.

That EFTs diminishes the fluorescence of dye-EFTu-GTP is required by the previous observations that the fluorescence of dye-EFTu-GTP is greater than that of dye-EFTu-GDP, and EFTs does not alter the fluorescence of dye-EFTu-GDP; therefore, these observations constitute a test of the consistency of the system. That EFTu in the EFTu-EFTs complex resembles EFTu-GDP rather than EFTu-GTP might have been expected, since EFTu when bound to EFTs should be in a form that does not bind AA-tRNA. Furthermore, displacement of GDP by EFTs would be facilitated if little or no conformational change were involved.

Discussion

Interaction of Dye with EFTu-GDP. The equilibrium dialysis data and the fluorescence data can be rationalized if one as-

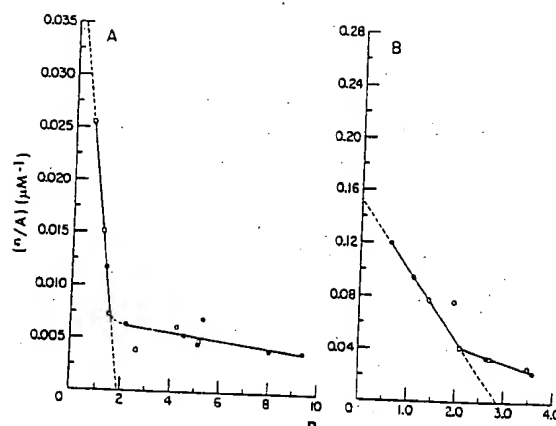


FIGURE 7: Scatchard plots, equilibrium dialysis of EFTu-GDP and EFTu-GTP with 1-anilino-8-naphthalenesulfonate (ANS); n , number of moles of dye bound per mole of EFTu, calculated on the basis of a molar extinction coefficient of 4.9×10^3 ; A, free dye; (A) EFTu-GDP; (O) [EFTu-GDP] = 9.9×10^{-5} M; (●) [EFTu-GDP] = 7.9×10^{-5} M. (B) EFTu-GTP; (O) [EFTu-GTP] = 3.0×10^{-5} M; (●) [EFTu-GTP] = 2.9×10^{-5} M.

sumes a difference in fluorescence yield between the two populations of binding sites. Thus, there are two sites of $K_{diss} = 5-8 \times 10^{-5}$ M with micromolar fluorescence yield of 70, corresponding to region a of the double reciprocal plot, and a very large number of other sites, corresponding to region b, which both bind and fluoresce much more weakly. The convex nature of the fluorescence Scatchard plot could arise from the fact that a constant, high fluorescence yield was used to calculate " n " whereas a decreasing, composite value of the fluorescence yield would be more valid.

Interaction of Dye with EFTu-GTP. The equilibrium dialysis data indicate that there are three equivalent dyes bound tightly ($K_{diss} = 1.9 \times 10^{-5}$ M) plus at least one other population of less tightly bound dyes. The fluorescence data indicate that the second dye molecule that interacts with EFTu-GTP has, in effect, a higher fluorescence yield than the first dye. In order to satisfy both the equilibrium dialysis data for site equivalency and the fluorescence data for site difference, it is necessary to postulate a kind of cooperativity of fluorescence, such that regardless which site is first occupied, giving rise to

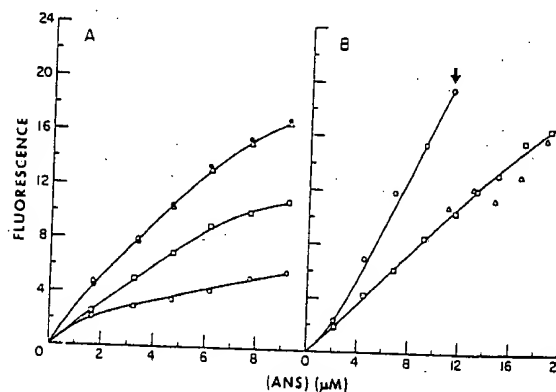


FIGURE 8: Effect of EFTs on the fluorescence of dye-EFTu-GDP and dye-EFTu-GTP: (A) titration of EFTu-GDP by dye in the presence and absence of EFTs: (□) EFTu-GDP, 1.3×10^{-5} M; (O) EFTs, 2×10^{-5} M (control curve); (Δ) summation of previous two curves; (●) EFTu-EFTs (EFTu-GDP and EFTs were combined prior to titration at the same concentrations as when titrated separately). (B) Effect of EFTs on the titration curve of dye-EFTu-GTP. At arrow, the solution being titrated was made 2×10^{-5} M in EFTs: (O) EFTu-GTP; (□) EFTu-GDP (control curve); (Δ) EFTu-GTP after addition of EFTs and correction for EFTs enhancement of fluorescence.

region a of Figure 4, the second and third sites will have an apparent higher fluorescence yield. This would result in the slope increase seen in region b of the double reciprocal plot and in the fluorescence Scatchard plot. The final decrease in slope of region c of Figure 4 would be due to the decreased fluorescence yield of the more weakly bound dyes indicated somewhat incompletely in the equilibrium dialysis data.

Conformational Differences between EFTu-GTP and EFTu-GDP as Evidenced by 1-Anilino-8-naphthalenesulfonate Binding. Although binding of AA-tRNA appears to cancel the difference between the two complexes in terms of their overall interaction with 1-anilino-8-naphthalenesulfonate, it is not possible to conclude that their conformational difference is localized to the AA-tRNA binding site. The observed differences in dissociation constant and fluorescence yield of the bound dye molecules could, however, be due to small perturbations in basically similar sites. Indeed, circular dichroic (CD) studies of EFTu-GDP and EFTu-GTP show no detectable differences in conformation (data not shown).

The assumption throughout this work has been that differential binding of the sulfonate by the two complexes truly reflected differences in conformation. The other possibility, that differential binding is due to selective binding to the nucleotides, is highly unlikely. Both complexes show a high and identical specificity for guanosine; close analogs such as the di- and triphosphates of inosine and xanthosine show no affinity for EFTu. Thus, the guanosine moiety is probably bound to the protein and is unavailable for interaction with the dye in both complexes. The additional phosphate moiety of GTP is also not likely to cause enhanced binding of the anion 1-anilino-8-naphthalenesulfonate.

Function of EFTu. Whereas the results from these experiments and the tritium exchange studies support the view that GTP induces EFTu to assume a conformation that selectively binds AA-tRNA, little is known about subsequent functions of EFTu-GTP. Whether the interaction of EFTu-GTP with AA-tRNA causes a significant alteration in the structure of the tRNA is uncertain. Nmr studies of the base-pair hydrogen bonds in AA-tRNA show that interaction with EFTu-GTP does not change the extent of base pairing in tRNA (C. Hilbert *et al.*, submitted for publication); however, changes in the tertiary structure of AA-tRNA are still possible. Although details of the reaction of the ternary complex with the ribosome remain unclear, it appears that the conformational change accompanying the hydrolysis of GTP to GDP allows EFTu to be removed from the ribosome, freeing the aminoacyl group for peptide bond formation. GDP is then displaced by EFTs with no apparent conformation change in EFTu; the cycle of reactions is complete when GTP interacts with the EFTu-EFTs complex to change the conformation of EFTu into its AA-tRNA binding form.

EFTu-GTP as a Model for the Function of Nucleoside Polyphosphates. The role of GTP in the function of EFTu resembles the role of other nucleoside polyphosphates in the function of motile protein systems and allosteric enzymes. As examples, GTP is an essential effector for CTP synthetase when glutamine is the nitrogen donor (Levitzki and Koshland, 1972) and CTP is an allosteric inhibitor of aspartate transcarbamoylase. These effects are thought to be transmitted to the active site by conformational changes induced by the allosteric ligand; however, other explanations must be considered. In the case of aspartate transcarbamoylase, CTP may inhibit the enzyme by a steric effect rather than a conformational alteration (Warren *et al.*, 1973). The validity of this proposal can be tested; however, it is unlikely that this hy-

pothesis can be extended to explain allosteric acceleration. Fluorescence studies have previously identified conformational changes induced by nucleotide allosteric effectors. In a study of the binding of 1-anilino-8-naphthalenesulfonate to phosphofructokinase, the allosteric effector AMP greatly decreased the fluorescence of the dye-phosphofructokinase complex.

Nevertheless, these enzymes are usually multisubunit complexes, and sometimes, as is true of aspartate transcarbamylase, the regulatory and catalytic sites are on different types of subunits. These properties complicate studies of conformational changes. Similarly, the proteins involved in motility, such as actomyosin, the dynein-tubulin complex of cilia, and the EFG-ribosome-mRNA complex are multi-protein aggregates where the primary function of ATP or GTP is extremely difficult to identify, although there is evidence that the triphosphates induce different conformational states than the diphosphates do (Schaub and Watterson, 1973; Cheung, 1969; Werber *et al.*, 1972).

Motile systems could be related to allosteric systems in that both processes could be initiated by a conformational change induced by a nucleoside triphosphate (Hill, 1969). The former process could be reversed by the dissociation of the inducer, whereas the motile system could be rendered unidirectional and irreversible by hydrolysis of the inducer together with additional interactions between the components after the hydrolytic step. This idea becomes more attractive with the demonstration that a nucleoside triphosphate can promote a significant conformational change upon binding to a relatively small, simple protein of one polypeptide chain.

References

- Beyer, C. F., Craig, L. C., and Gibbons, W. A. (1972) *Biochemistry* 11, 4920.
- Bloxham, D. (1973), *Biochemistry* 12, 1602.
- Brand, K. (1970), *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 235.
- Brand, L., and Gohlke, J. R. (1972), *Annu. Rev. Biochem.* 41, 843.
- Cheung, H. C. (1969), *Biochim. Biophys. Acta* 194, 478.
- Chuang, D., and Simpson, M. V. (1971), *Proc. Nat. Acad. Sci. U. S.* 68, 1474.
- Daniel, E. and Weber, G. (1966), *Biochemistry* 5, 1893.
- Gillam, I. C., and Tener, G. M. (1971), *Methods Enzymol.* 20, 55.
- Hill, T. L. (1969), *Proc. Nat. Acad. Sci. U. S.* 64, 267.
- Levitzki, A., and Koshland, D. E. (1972), *Biochemistry* 11, 241.
- Lucas-Lenard, J., and Lipmann, F. (1971), *Annu. Rev. Biochem.* 40, 409.
- Miller, D. L., Cashel, M., and Weissbach, H. (1973), *Arch. Biochem. Biophys.* 154, 675.
- Miller, D. L., and Weissbach, H. (1970), *Arch. Biochem. Biophys.* 141, 26.
- Miller, D. L., and Weissbach, H. (1974), *Methods Enzymol.* 30, 219.
- Moffatt, J. G., and Khorana, H. G. (1961), *J. Amer. Chem. Soc.* 83, 649.
- Printz, M. P., and Miller, D. L. (1973), *Biochem. Biophys. Res. Commun.* 53, 149.
- Scatchard, G., Colema, J. S., and Shen, A. L. (1956), *J. Phys. Chem.* 79, 12.
- Schaub, M. C., and Watterson, J. G. (1973), *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 30, 305.

1 of Braun

this should
by a journal
as any other
est estimate
full citation

he press.
d in a type
at it will be
stage before
hen the name

mmunication
al value.
l work should
of references
the former.

ical Abstracts
Edition. The
ion available
Abbreviations
ng Group on
the abbrevia-
U will be very
cts.

this issue is
may be a few
e Commission
ould now use
ciety) system
at will be re-
endations on

ations

on, May 7-8
of uniformly
desirable and
mical journals
on as possible
will have to
e minor) from
plement these
t the possible
rcial publisher
eighed by the
tion of referen-
readers upon

al Abstracts are
List of Serials
Sciences Inter-
0 Arch Street

Kinetic Properties of Phenylalanyl-tRNA and Seryl-tRNA Synthetases for Normal Substrates and Fluorescent Analogs

Harry S. HERTZ and Hans G. ZACHAU

Institut für Physiologische Chemie und Physikalische Biochemie der Universität München

(Received March 28, 1973)

The kinetics of phenylalanyl-tRNA and seryl-tRNA formation were investigated with tRNAs and aminoacyl-tRNA synthetases from yeast.

Phenylalanyl-tRNA synthetase yielded linear Lineweaver-Burk plots with tRNA^{Phe}, phenylalanine, and 1,N⁶-ethenoadenosine triphosphate (ϵ ATP) as variable substrates. According to equilibrium dialysis in the absence or presence of phenylalaninyl adenosine 5'-phosphate, phenylalanyl-tRNA synthetase possesses one binding site for phenylalanine. For ATP as variable substrate, the deviation from linearity in the Lineweaver-Burk plot, observed by other investigators, was confirmed. The slope of the curve indicates the presence of more than two ATP binding sites.

Seryl-tRNA synthetase yielded a linear Lineweaver-Burk plot only with ϵ ATP as variable substrate. The Lineweaver-Burk plots for serine and tRNA^{Ser} were non-linear; the interpretation we favor involves positive cooperativity between amino acid binding sites and between tRNA binding sites. Hill plots of the kinetic data showed that the enzyme possesses at least two binding sites for each of these substrates. The kinetic data for ATP could be interpreted as showing more than two binding sites with negative and positive cooperativity in binding of successive ATP molecules.

The aminoalkyl adenylates, phenylalaninyl adenosine 5'-phosphate and serinyl adenosine 5'-phosphate, competitively inhibited the aminoacylation reaction with respect to amino acid.

ϵ ATP functions in place of ATP in phenylalanyl-tRNA and seryl-tRNA formation although with rather different kinetic properties. Modified tRNA^{Phe} and tRNA^{Ser}, in which the 3'-terminal adenosine was replaced by ethenoadenosine, were prepared by a C-C-A transferase-catalyzed reaction of ϵ ATP. These modified tRNAs show kinetic properties very similar to those of the unmodified tRNAs and can therefore be used, in place of the unmodified tRNAs, as fluorescent probes in synthetase-tRNA interaction studies.

The kinetic behavior of phenylalanyl-tRNA synthetase appears to be much simpler than that of seryl-tRNA synthetase, despite the fact that the former enzyme is twice as big and contains twice as many subunits as the latter one. The comparative simplicity of the one enzyme relative to the other correlates with previous results on interactions with substrates, which were obtained by fluorescence measurements and nuclease protection studies.

Dedicated to Professor A. Butenandt on the occasion of his 70th birthday.

Abbreviations. ϵ ATP, 3- β -D-ribofuranosylimidazo[2,1-f]purine 5'-triphosphate, or 1,N⁶-ethenoadenosine triphosphate; tRNA_A, tRNA which has its 3'-terminal adenosine replaced by ethenoadenosine; Phe-ol-pA, phenylalaninyl adenosine 5'-phosphate; Ser-ol-pA, serinyl adenosine 5'-phosphate.

Enzymes. Phenylalanyl-tRNA synthetase (EC 6.1.1.-); seryl-tRNA synthetase (EC 6.1.1.11); C-C-A transferase or C-C-A pyrophosphorylase or nucleoside triphosphate:tRNA nucleotidyltransferase (EC 2.7.7.25); snake venom phosphodiesterase (EC 3.1.4.1).

Definitions. $A_{260(280)}$ unit, the quantity of material contained in 1 ml of a solution which has an absorbance of 1 at 260 (280) nm, when measured in a cell with 1-cm pathlength; " K_m ", the apparent Michaelis constant obtained in non-linear Lineweaver-Burk plots by extrapolation of the data at high substrate concentration to the 1/[S] axis.

The mechanism and specificity of action of aminoacyl-tRNA synthetases are of considerable current interest (Summaries, e.g. [1,2]). In this laboratory we have concerned ourselves with the phenylalanyl-tRNA and seryl-tRNA synthetases from yeast. Phenylalanyl-tRNA synthetase is a tetramer of the $\alpha_2\beta_2$ type, with a molecular weight of about 240 000 [3] and seryl-tRNA synthetase is a dimer with a molecular weight of about 120 000 [4-6]. The two synthetases have already been the subject of rather extensive studies in this laboratory. The interactions between the synthetases and their substrates have been investigated by fluorescence methods [4,5,7]. Some information on the topology of the synthetase-tRNA complexes was obtained by partial nuclease digestion

[8]. The regions of the tRNA molecules which are essential for aminoacylation by synthetases were defined by numerous methods, for example through studies of fragments of tRNA^{Phe} [9] and tRNA^{Ser} [10].

The purpose of the present investigation was to complement the interaction studies by a detailed examination of the aminoacylation kinetics of tRNA^{Ser} and tRNA^{Phe} and of fluorescent derivatives of these tRNAs. These derivatives, tRNA^{Ser}_{εA} and tRNA^{Phe}_{εA}, were prepared by a C-C-A transferase-catalyzed reaction of 1, *N*⁶-ethenoadenosine 5'-triphosphate (εATP), which had been synthesized according to Barrio *et al.* [11]. The comparison of the kinetic parameters of the fluorescent derivatives with those of the natural substrates is important for the evaluation of fluorometric binding studies.

MATERIALS AND METHODS

tRNAs and Enzymes

tRNA^{Phe} and tRNA^{Ser} were prepared from baker's and brewer's yeasts [12,13]. The acceptor activities of the tRNAs from baker's yeast are listed in Table 1. If not otherwise stated tRNA^{Phe} and tRNA^{Ser} from brewer's yeast were used. These tRNAs accepted in the standard aminoacylation assay [14] more than 1.1 nmol phenylalanine/*A*₂₆₀ unit and 1.0 nmol serine/*A*₂₆₀ unit, respectively.

Phenylalanyl-tRNA synthetase and seryl-tRNA synthetase from yeast were prepared in cooperation with R. Hirsch as described previously [3–5,15] (and R. Hirsch, unpublished work) and had the previously described properties [4,5]; some samples were generously provided by R. Hirsch. One *A*₂₆₀ unit phenylalanyl-tRNA synthetase and seryl-tRNA synthetase were taken to be 1 mg and 0.82 mg, respectively. C-C-A transferase (0.026 units [16]/*A*₂₆₀ unit) was a gift from H. Overath and snake venom phosphodiesterase was obtained from Boehringer-Mannheim GmbH (Mannheim, W. Germany).

[¹⁴C]Phenylalanine and [¹⁴C]serine were products of the Radiochemical Centre (Amersham). Na₂ATP was purchased from Papierwerke Waldhof-Aschaffenburg (Mannheim, W. Germany).

Preparation of Aminoalkyl Adenylates

Phenylalaninyl adenosine 5'-phosphate (Phe-ol-pA, I) was prepared according to the method of Sandrin and Boissonnas [17].

Serinyl adenosine 5'-phosphate (Ser-ol-pA, II): the starting material, *N*-(*t*-butoxycarbonyl)-*O*-(*t*-butyl)-serine dicyclohexylammonium salt was prepared by F. Drees and E. Wünsch through acylation of *O*-(*t*-butyl)-serine [18] and was kindly donated. 2.2 g (5 mmol) of this compound was converted to the free acid [19] and esterified with diazomethane.

Table 1. Amino-acid acceptance of baker's yeast tRNAs. Amino acid incorporation was at 37 °C, for 20 min with purified synthetases, otherwise as previously described [14]. 0.1 mU C-C-A transferase was used per 0.1 ml incubation mixture

Substance tested	C-C-A transferase	Incorporation of amino acid per <i>A</i> ₂₆₀ unit	Incorporation
		%	%
tRNA ^{Phe}	+	950	100
tRNA ^{Phe} _{εA}	—	25	2.6
tRNA ^{Ser}	+	900	95
tRNA ^{Ser} _{εA}	—	1180	100
tRNA ^{Ser}	—	20	1.5
tRNA ^{Ser} _{εA}	—	840	71

0.54 g (2.0 mmol) of the ester was dissolved in 30 ml absolute tetrahydrofuran and 2.5 g (>0.1 mol) LiBH₄ was added with constant stirring and outer cooling (similar to [20]). The reaction mixture was heated for 24 h at 75–80 °C under reflux conditions with exclusion of moisture. After cooling, 20 ml of a water-saturated *n*-butanol solution were added and the mixture stirred until gas evolution ceased. The precipitate was filtered and boiled three times with 30 ml *n*-butanol. The combined solutions were concentrated to dryness, the residual oil dissolved in 20 ml ether and extracted three times with 10 ml water each. The ether solution was then dried over anhydrous sodium sulfate and evaporated to dryness to yield 0.358 g (1.45 mmol) of *N*-(*t*-butoxycarbonyl)-*O*-(*t*-butyl)-serinol. The structure of this compound and the previous ester were confirmed by mass spectrometry.

The serinol derivative was further reacted with *N*,*O*^{2'},*O*^{3'}-triacyl-adenosine-5'-phosphate [21] according to the procedure of Sandrin and Boissonnas [17], to yield 0.126 g (0.22 mmol) *N*-(*t*-butoxycarbonyl)-*O*-(*t*-butyl)-serinyl adenosine 5'-phosphate. The oily product was homogeneous on silica-gel thin-layer chromatography using acetone–water (8:2 v/v) as the solvent system (detection by ultraviolet and periodate-benzidine [22]).

The protecting groups were removed by allowing the product to react 5 min at room temperature in 1 ml trifluoroacetic acid and then evaporating to dryness on a rotary evaporator. Thin-layer chromatography using the same conditions as above revealed three major spots, of which only the one with the lowest *R*_F value (0.25) was both ninhydrin and periodate-benzidine positive. 5.4 mg (0.013 mmol) of this compound were isolated by kieselgel column chromatography, using acetone–water (8:2, v/v) as the eluent.

To confirm the structure of the Ser-ol-pA, 0.3 *A*₂₆₀ units in 0.1 M sodium cacodylate buffer pH 7.2 containing 10 mM MgCl₂ were digested with 6.4 units [23] snake venom phosphodiesterase for 45 min.

37 °C
thin.
(8:2
(18:
togr
serin
com]

of B
aceta
W. G
prepe
units
yeast
were
for 30
8 ml
and 2
NaOH
was d
on a l
ly ec
sodium
The
absor
then
to ob

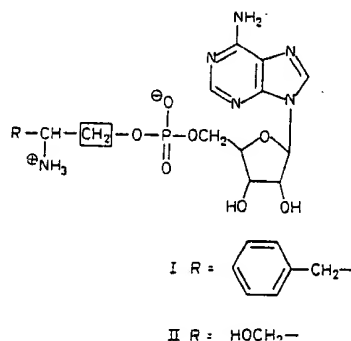
In
varial
6–7 I
were
by ad
subst
or 10
and p
precip
filters
[14].
such t
linear

1st tRNAs
20 min with
described [14].
al incubation

Incorporation

%
100
2.6
95
100
1.5
71

37 °C and then applied directly to two silica-gel thin-layer plates, which were run in an acetone-H₂O (8:2, v/v) and a methanol-water-acetic acid (18:2:0.1, v/v/v) system, respectively. The chromatograms revealed cleavage of the product to serinol and adenosine 5'-phosphate, as shown by comparison with authentic samples.



ϵ ATP, tRNA_{εA}

ϵ ATP was prepared according to the procedure of Barrio *et al.* [11] with the exception that chloroacetaldehyde hemihydrate (Schuchardt, München, W. Germany) was used as starting material. For the preparation of tRNA_{εA}^{Phe} and tRNA_{εA}^{Ser} 50 A₂₆₀ units of each of these tRNAs isolated from baker's yeast (*i.e.* tRNAs lacking the 3'-terminal AMP) were incubated with 8.6 mU [16] C-C-A transferase for 30 min at 37 °C. Each reaction was carried out in 8 ml of a 12 mM ϵ ATP, 18 mM NH₄Cl, 18 mM MgCl₂, and 30 mM Tris-HCl solution, adjusted to pH 7 with NaOH. After the 30-min reaction time, the mixture was diluted with an equal volume of water and loaded on a DEAE-Sephadex column (0.6 × 10 cm) previously equilibrated with the following buffer: 20 mM sodium acetate, 12.5 mM MgCl₂, 0.3 M NaCl, pH 5.2. The column was washed with the buffer until the absorbance at 260 nm returned to baseline level and then eluted with buffer containing 1 M NaCl in order to obtain the tRNA_{εA}^{Phe} or tRNA_{εA}^{Ser}.

Aminoacylation Kinetics

Initial velocity values at each concentration of a variable substrate were based on time curves of 6–7 points per concentration. The kinetic experiments were performed at 24 °C and reactions were started by addition of enzyme to a solution containing the substrates. For each data point on a time curve 50 or 100 μl of incubation mixture were withdrawn and pipetted into cold 5% trichloroacetic acid. The precipitate was filtered on glass fiber filters and the filters prepared for counting as previously described [14]. Times and enzyme concentrations were chosen such that one was always operating in the range of linear rate dependence (between 2 and 7 min maxi-

mum). The thus-determined initial velocity values were then plotted as a function of substrate concentration in Lineweaver-Burk plots [24].

Kinetic studies with phenylalanyl-tRNA synthetase (0.12–0.17 μg/ml incubation mixture) were performed in 50 mM Tris-HCl pH 7.5, 15 mM MgCl₂, 0.3 μM bovine serum albumin and 4 mM glutathione (reduced). Unless otherwise specified, substrate concentrations were as follows: 5 mM ATP, 38–50 μM phenylalanine, and 4 μM tRNA^{Phe}.

Kinetic studies with seryl-tRNA synthetase (0.42–0.85 μg/ml incubation mixture) were performed in 50 mM Tris-HCl pH 7.5, 0.3 μM bovine serum albumin, 4 mM glutathione (reduced) and 100 mM KCl. Unless otherwise specified, substrate concentrations were as follows: 25 mM ATP, 30 mM MgCl₂, 44–60 or 220 μM serine, and 4 or 16 μM tRNA^{Ser}.

Equilibrium-Dialysis Experiments

Equilibrium dialyses were carried out at 4 °C in a lucite cell. Both chambers were 100 μl in size. Visiting dialysis membranes, which had been boiled in water and soaked in the dialysis buffer, were employed. The buffer was 50 mM Tris-HCl pH 7.5, 5 mM MgCl₂, 0.5 mM dithiothreitol and 100 mM KCl. Each chamber was filled with 50 μl of solution, one containing the aminoacyl-tRNA synthetase and cognate tRNA and the other the ¹⁴C-labeled substrate being investigated.

Dialyses ran for 5–18 h, times which were previously shown to be sufficient for complete equilibration. At each of two time points three 5-μl samples from the chambers were pipetted into vials containing 5 ml of scintillation fluid (4 g Omnifluor dissolved in 1 l of 1:3 mixture of Triton X-100/toluene) and counted in a Packard Tri-Carb liquid scintillation spectrometer.

RESULTS

tRNA_{εA}

tRNA_{εA}^{Ser} and tRNA_{εA}^{Phe} were prepared from the corresponding baker's yeast tRNAs. According to aminoacylation experiments (Table 1), more than 95% of the tRNA molecules lacked the 3'-terminal AMP. Cytosine was completely present according to incorporation experiments (not shown). tRNA_{εA}^{Phe} can be aminoacylated to the same extent as tRNA^{Phe} from baker's yeast, to which the terminal AMP has been added during the aminoacylation reaction. tRNA_{εA}^{Ser} accepted approximately 70% as much serine as the adenosine-containing tRNA^{Ser}. Experiments showed that the C-C-A transferase was not limiting in the incubation mixture; no attempts were made to obtain a tRNA_{εA}^{Ser} preparation with full amino acid acceptor activity.

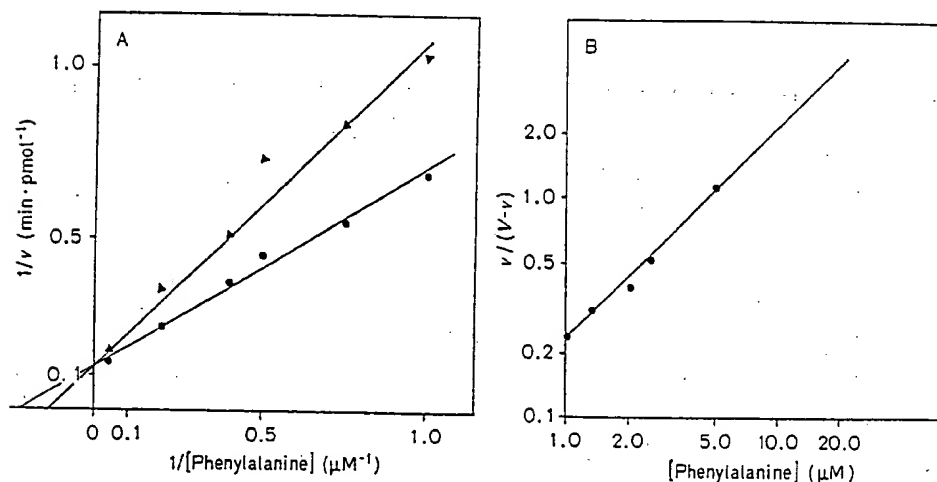


Fig. 1. Determination of kinetic parameters for Phe-tRNA^{Phe} formation with phenylalanine as the variable substrate. (A) Determination of \bar{V} and K_m for phenylalanine (●—●), and the inhibitory effect of Phe-ol-pA on the aminoacylation

reaction (▲—▲). For the inhibition studies 0.1 μM Phe-ol-pA was present. (B) Determination of the order of the reaction with respect to phenylalanine, according to the empirical Hill equation (see text)

Table 2. Results of kinetic experiments with phenylalanyl-tRNA synthetase

For details of the conditions see Methods. For each substance the concentration range tested is specified. For Phe-ol-pA studies, a fixed concentration of the inhibitor was used, and amino acid concentration was varied as in the experiments without the inhibitor. n values are the interaction constants obtained from Hill plots (see text)

Substance	Concentration μM	K_m or " K_m " μM	K_i μM	n	\bar{V} $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$
Phenylalanine	1–25	4.4		0.97	varied between 0.26–0.57 depending on the batch of enzyme
Phe-ol-pA	0.1		0.16		
tRNA ^{Phe}	0.1–5	0.12		0.97	
ATP	50–10000	800			
tRNA ^{Phe}	0.033–5	0.055		1.0	0.18
ϵATP	200–10000	2220		0.98	0.18

The fluorescence emission spectra of tRNA^{Ser} and tRNA^{Phe} are the same as that of ϵATP [11,25]. A corrected emission spectrum of tRNA^{Ser} is presented elsewhere [7]. Results of kinetic experiments with the modified tRNAs are presented below.

Kinetic Studies with Phenylalanyl-tRNA Synthetase

The kinetics of the formation of Phe-tRNA^{Phe} were reasonably straightforward. Standard conditions were employed with no new effort to optimize them. K_m and \bar{V} values were obtained by the method of Lineweaver and Burk [24]. Interaction constants (n values) were determined from plots of reaction velocity vs substrate concentration according to the empirical Hill equation [26,27]. The results of the various experiments are summarized in Table 2.

Results of experiments with Phe-ol-pA (Fig. 1A) show that it is a potent inhibitor ($K_i = 0.16 \mu\text{M}$), which competitively inhibits binding of phenylalanine ($K_m = 4.4 \mu\text{M}$) to its site on phenylalanyl-tRNA synthetase. A Hill plot (Fig. 1B) of the

kinetic data for aminoacylation, with phenylalanine as the variable substrate, gives an n value of 0.97.

A comparison of the data for tRNA^{Phe} and tRNA^{Ser} (Fig. 2) shows that tRNA^{Phe} has both a lower K_m and leads to a lower \bar{V} for the aminoacylation reaction than is observed with tRNA^{Ser}. Apparently the modified tRNA is bound somewhat tighter to the enzyme and is, under saturating conditions, aminoacylated somewhat slower than the unmodified tRNA.

Kinetic experiments with ATP as limiting substrate (Fig. 3A) yielded linear Lineweaver-Burk plots in the concentration range 0.5–10 mM. Following unpublished results of Berther, Mayer and Dutler we then also observed a deviation from linearity at low ATP concentrations. For this non-linear reciprocal plot and for those obtained with seryl-tRNA synthetase (see below) we have introduced " K_m " and defined it as the apparent Michaelis constant obtained by extrapolation of the data at high substrate concentration to the $1/[S]$ axis in the Lineweaver-Burk plot.

Kinetics of the aminoacylation reaction with ϵ ATP as variable substrate yielded a linear Lineweaver-Burk plot in the concentration range 0.2 to 10 mM (Fig. 3B). Attempts to obtain accurate rate determinations at lower ϵ ATP concentration than 0.2 mM were unsuccessful.

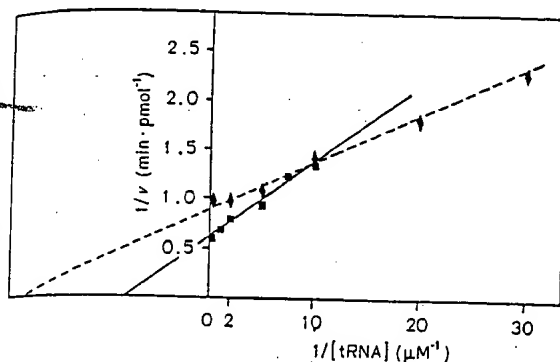


Fig. 2. Lineweaver-Burk plot comparing $tRNA^{Phe}$ (■—■) and $tRNA^{Ala}$ (◆—◆) as substrates of phenylalanyl-tRNA synthetase in the aminoacylation reaction.

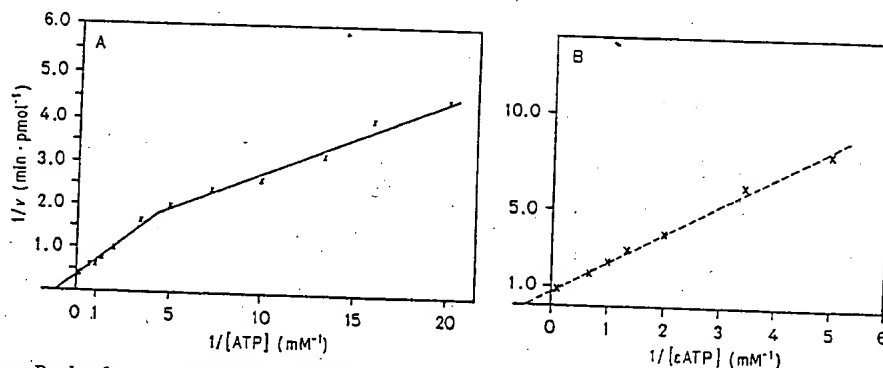


Fig. 3. Lineweaver-Burk plots of kinetic data obtained with phenylalanyl-tRNA synthetase and (A) ATP or (B) ϵ ATP as variable substrate.

Table 3. Results of kinetic experiments with seryl-tRNA synthetase

For details of conditions see Methods. For each kinetic experiment the substance concentration range tested, as well as the fixed concentrations of $tRNA^{Ser}$ and serine are specified. For Ser-ol-pA studies, a fixed concentration of the inhibitor was used, and amino acid concentration was varied as in the experiments without the inhibitor. n values are the interaction constants obtained from Hill plots (see text).

Substance	Concentration μM	$tRNA^{Ser}$ μM	Serine μM	K_m or " K_m " μM	" K_i " μM	n	v $\mu mol \cdot min^{-1} \cdot mg \text{ protein}^{-1}$
Serine	37.5–1000	4	—	40	—	1.78	0.20–0.44
Ser-ol-pA	0.84	4	—	—	0.6	—	—
$tRNA^{Ser}$	0.092–4.0	—	60	0.091	—	1.77	0.11
$tRNA^{Ser}$	0.13–1.0	—	220	0.38	—	1.53	0.21–0.41
ATP	12.5–20000	4	44	1500	—	—	0.11
ATP	12.5–20000	4	220	1700	—	—	0.23–0.44
$tRNA^{Ser}$	12.5–20000	16	44	2300	—	—	0.11
ϵ ATP	0.077–1.0	—	60	0.033	—	1.64	0.10
ϵ ATP	25–20000	4	44	1000	—	0.93	0.05

Kinetic Studies with Seryl-tRNA Synthetase

As can be seen from the Lineweaver-Burk plots (Fig. 4–6), seryl-tRNA synthetase does not exhibit normal Michaelis-Menten kinetics. Care was therefore taken to optimize reaction conditions before detailed kinetic investigations were undertaken. The maximum rate of aminoacylation was found at a salt concentration of 80–120 mM KCl, and at a 5–10 mM excess of Mg^{2+} over ATP, which was optimized at 25 mM. Results of kinetic experiments with seryl-tRNA synthetase are summarized in Table 3.

Kinetic experiments with Ser-ol-pA (Fig. 4) indicate that it competitively inhibits the aminoacylation reaction with respect to serine. In this case it was particularly difficult to obtain smooth curves. From a number of independent experiments it appears, however, that the shapes of the curves were very similar to the ones obtained in the absence of inhibitor. Therefore the conclusion of competitive inhibition seemed justified. In keeping with our definition of " K_m " (see above), we have defined " K_i " as the inhibition constant obtained from the ratio of slopes at high concentration, in the Line-

weaver-Burk plot. Thus defined, serine has a " K_m " of 40 μM and Ser-ol-pA a " K_i " of 0.6 μM .

Kinetic experiments with tRNA^{Ser} as variable substrate were performed at two amino acid concentrations. Only the experiments with 220 μM serine (Fig. 5B) were in the region of amino acid saturation

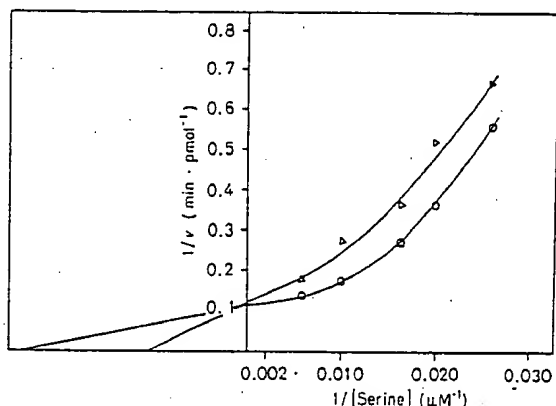


Fig. 4. Determination of V and " K_m " for serine (○—○), and the inhibitory effect of Ser-ol-pA on the aminoacylation reaction (Δ—Δ). For the inhibition studies 0.84 μM Ser-ol-pA was present

and yielded a maximal V value. The experiments with 60 μM serine (Fig. 5A) gave a lower V . In addition, the " K_m " for tRNA^{Ser} was different at the two serine concentrations. The kinetic data obtained at 60 μM serine were evaluated by a Hill plot (Fig. 5C) and yielded an interaction constant of 1.77. A comparison of the kinetic data obtained with tRNA^{Ser} and tRNA^{Ser} , both at 60 μM serine, shows that tRNA^{Ser} has a slightly lower " K_m " value and that the V value obtained with the two substrates is about the same.

A plot of $1/v$ vs $1/[S]^2$ [29] yields a straight line for the data with tRNA^{Ser} (Fig. 5D) and serine (not shown) as the variable substrates. This is in agreement with the parabolic, concave-upwards, appearance of the corresponding Lineweaver-Burk plots and confirms the quadratic dependence of substrate in the double-reciprocal plots.

Kinetic experiments with ATP as variable substrate were carried out at various fixed concentrations of serine and tRNA^{Ser} (Fig. 6A—C). These variations in the fixed substrate concentrations have little effect on the " K_m " value. The V value for seryl-tRNA synthetase is, as above, raised by increasing the serine concentration from 44 to 220 μM .

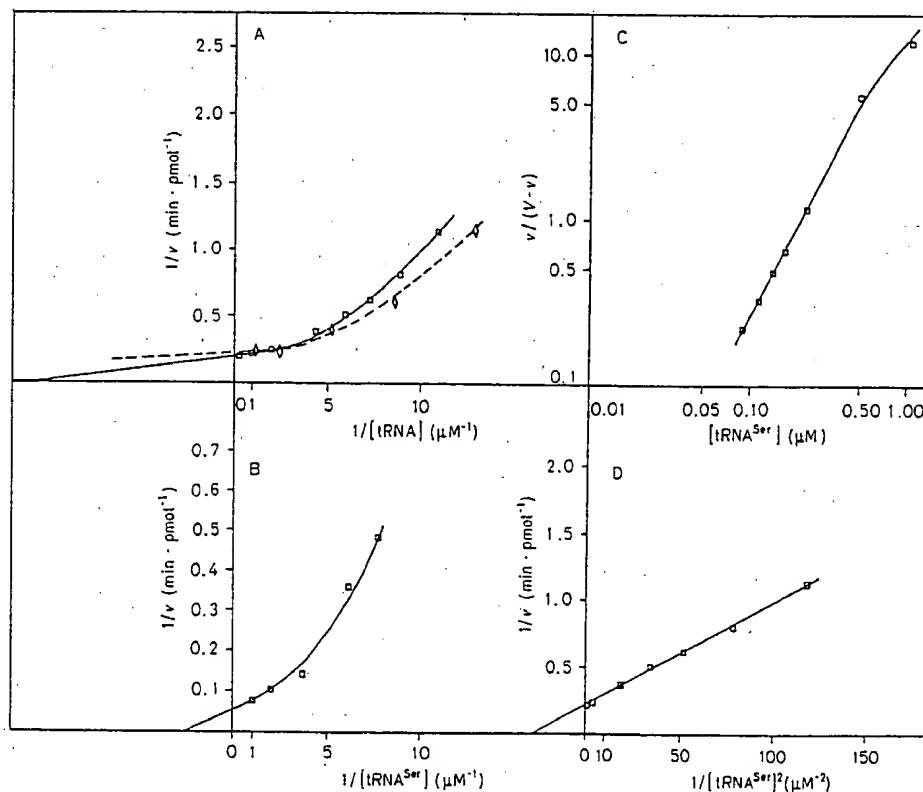


Fig. 5. Results of kinetic studies with tRNA^{Ser} or tRNA^{Ser} as variable substrate in the aminoacylation reaction. (A) Lineweaver-Burk plot, 60 μM serine, tRNA^{Ser} (□—□) or tRNA^{Ser} (○—○) as variable substrate; (B) Lineweaver-

Burk plot, 220 μM serine, tRNA^{Ser} as variable substrate; (C) determination of reaction order with respect to tRNA^{Ser} (data from A), according to the Hill equation; (D) plot of $1/v$ vs $1/[S]^2$ for the tRNA^{Ser} data in (A)

Fig. 6. μ plots for

only t velocity at 4 μM to 16 μM interest various ATP, (Fig. 6) Michael a lower substrate

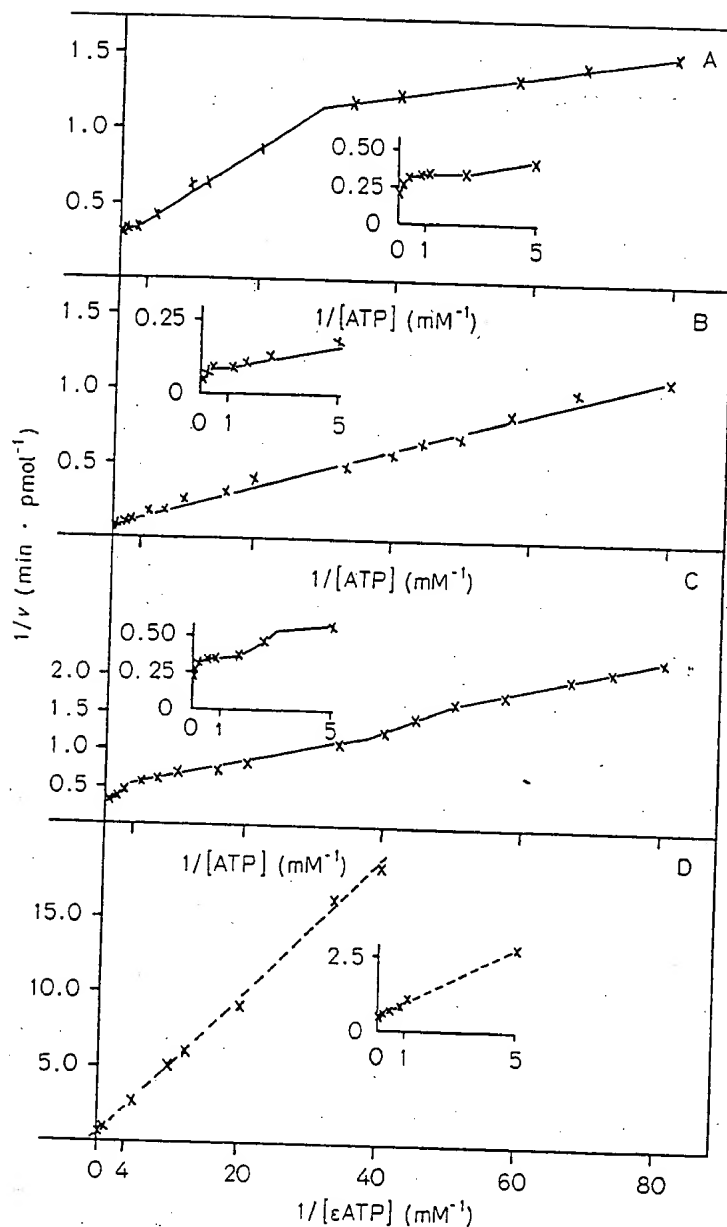


Fig. 6. Results of kinetic experiments with seryl-tRNA synthetase and ATP or ϵ -ATP as the variable substrate. Lineweaver-Burk plots for ATP with: (A) 4 μ M tRNA^{ser} and 44 μ M serine, (B) 4 μ M tRNA^{ser} and 220 μ M serine, and (C) 16 μ M tRNA^{ser} and 44 μ M serine. (D) Lineweaver-Burk plot for ϵ -ATP with 4 μ M tRNA^{ser} and 44 μ M serine

only the latter value representing a true maximal velocity. Increasing the tRNA concentration, which at 4 μ M is already in the region of substrate saturation to 16 μ M had no effect on V . A point of considerable interest is the change in shape of the curves at the various fixed substrate concentrations. In contrast to ATP, when ϵ -ATP was used as variable substrate (Fig. 6D) the enzyme seemed to follow normal Michaelis-Menten kinetics, with a K_m of 1.0 mM and a lower V , than was obtained with the unmodified substrate.

Equilibrium-Dialysis Studies with Phenylalanyl-tRNA and Seryl-tRNA Synthetases

As a supplement to the data obtained from the kinetic studies, an attempt was made to determine, by equilibrium dialysis, the number of binding sites on the enzyme for amino acid. In order to approach the conditions of the aminoacylation reaction, the amino acid was dialyzed against enzyme plus tRNA. The molar ratio of tRNA to enzyme was 2:1 and 1:1 for the serine and phenylalanine systems, respectively. The activities of the synthetases were the same

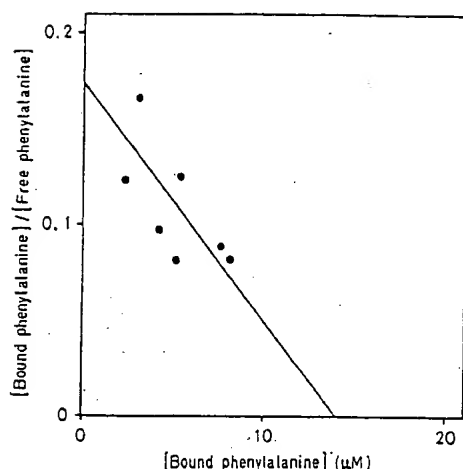


Fig. 7. Scatchard plot of results of the equilibrium dialysis of *L*-phenylalanine with phenylalanyl-tRNA synthetase ($16 \mu\text{M}$) and tRNA^{Phe} ($16 \mu\text{M}$)

before and after dialysis. A Scatchard plot [30] for the data with phenylalanyl-tRNA synthetase is presented in Fig. 7. The scatter of the points should be less at higher synthetase concentrations, but the data in Fig. 7 are sufficient to indicate a single binding site for phenylalanine per synthetase molecule with a binding constant of 10 mM^{-1} . The presence of a single binding site for phenylalanine was proven in an equilibrium dialysis experiment in which an equimolar amount of synthetase and Phe-ol-pA was employed. The Phe-ol-pA, the K_1 of which is 30-fold lower than the K_m of phenylalanine, prevented amino acid binding to the synthetase completely. At enzyme concentrations up to 9.4 mg/ml , no amino acid binding was evident with seryl-tRNA synthetase. Preliminary equilibrium dialysis experiments have been performed to determine the number of binding sites for ATP on seryl-tRNA synthetase. Also here a tRNA-to-enzyme ratio of 2:1 was used. The results indicate at least two binding sites for ATP.

DISCUSSION

Detailed analysis of the kinetic properties of aminoacyl-tRNA synthetases should yield some information on the mechanism of synthetase-substrate interactions. Furthermore, a comparison of the kinetic properties of natural and modified substrates is valuable when modified substrates are being used in other interaction studies, such as fluorescence binding studies. With phenylalanyl-tRNA synthetase normal Michaelis-Menten kinetics were generally observed, the K_m values lying in the concentration ranges reported for other aminoacyl-tRNA synthetases (see, e.g. [1, 2, 31, 32]). With seryl-tRNA synthetase, on the other hand, normal Michaelis-Menten kinetics

were not obtained. Since non-linear reciprocal plots were observed here, particular care was taken to exclude any stray effects. All curve forms reported were verified through multiple experiments and are completely reproducible.

Studies on Seryl-tRNA Synthetase

Before performing detailed kinetic experiments, the conditions for the seryl-tRNA synthetase-catalyzed aminoacylation reaction were optimized. The experimental parameters were found to differ considerably from the standard test conditions [4, 33]. By carrying out kinetic experiments in a buffer containing 100 mM KCl and a 5 mM excess of Mg^{2+} over ATP (25 mM) a significant increase in V was obtained. At saturating substrate concentrations and 24°C , values of 0.20 – $0.44 \mu\text{mol} \times \text{mg protein}^{-1} \times \text{min}^{-1}$ (corresponding to a specific enzyme activity of 0.20 – 0.44 International Units and a molecular activity of 24 – $53 \text{ mol} \times \text{mol}^{-1} \times \text{min}^{-1}$) were determined. It has been shown under standard test conditions [4] that V values obtained at the enzyme concentration required for the initial rate studies can be approximately doubled when the enzyme concentration range is raised above 10 nM [5].

With the optimized buffer conditions, studies of the kinetics of $\text{Ser-tRNA}^{\text{Ser}}$ formation yielded non-linear reciprocal plots (Fig. 4–6). The data for tRNA^{Ser} and serine as limiting substrates showed quadratic dependence and became linear when $1/[S]^2$ was plotted on the abscissa (Fig. 5D). The curves for ATP showed a more complex dependence and could not be simply linearized. Before discussing the implications of the data, a few comments about the theory of such non-linear curves is appropriate.

Non-linear reciprocal plots occur when isoenzymes or different active conformers of one enzyme are present, when one enzyme has two or more independent binding sites with different Michaelis constants, or when an enzyme exhibits cooperative binding effects (for a brief discussion, see [34]). The last possibility is discussed in more detail. A simple mathematical derivation of curved reciprocal plots in the case of two-place enzymes is presented elsewhere [35]. Cooperative binding interactions may be negative (reciprocal plot is concave downward) or positive (reciprocal plot is concave upward) corresponding to substrate activation or substrate inhibition of the rate of the catalytic reaction. In cases of positive cooperativity the binding of one substrate molecule eases the binding of the next substrate molecule. In cases of negative cooperativity, binding of one substrate molecule to the enzyme hinders the binding of the next molecule. However, a recent paper by Engel and Ferdinand [36] shows that curves with the same general reciprocal plot as seen in negative cooperativity can mathematically

be explained by negative cooperativity followed by positive cooperativity affecting the catalytic rate constant. In reciprocal plots containing only one transition the question of only negative or negative-plus-positive cooperativity requires detailed knowledge of the abruptness of change between the two regions, a smooth concave curve signifying the first alternative and an abrupt change the second alternative.

The existence of consecutive negative and positive cooperativity in the binding of one ligand to an enzyme has already been mathematically substantiated by Teipel and Koshland [37]: if the rate of equilibration between substrate and enzyme is rapid relative to the rate of catalysis, kinetic saturation curves possessing an intermediate plateau region can sometimes be explained by more than two substrate binding sites, with the relative magnitude of the substrate binding or catalytic constants first decreasing and then increasing as the enzyme sites are occupied. The total number of such sites could be contained in several enzyme conformers or subunits with different affinities.

In addition to results obtained from the Lineweaver-Burk plots, the data from kinetic experiments have been evaluated according to the empirical Hill equation. The value n obtained from the slope of the Hill plot was denoted as an interaction constant by Changeux [38]. Assuming a single active enzyme conformer, the slope is a measure of the strength of interaction between binding sites. Hence, a value (see Table 3) of 1 signifies one substrate binding site or several independent binding sites. A value between 1 and 2 implies that at least two interacting active sites are present on the enzyme. The stronger the interaction between the sites, the closer the n value will come to the number of binding sites. Therefore, on an enzyme with cooperative binding sites the difference between n and the number of sites is a measure of the degree of cooperativity: the smaller the difference the higher the degree of cooperativity. At high and low substrate concentrations the slope of the Hill plot will curve off from the high n value and tend to flatten out with limiting slopes of 1 at either end. This is visible in Fig. 5C where the curve gets flatter at the high log [tRNA^{Ser}] values.

With the above concepts in mind, at least a partial interpretation of the kinetic data for Ser-tRNA^{Ser} formation is possible. For this interpretation, we favor arguments involving cooperativity of binding sites although, of course, one cannot rule out the other possibilities mentioned above. Based upon the reciprocal plots, it would appear that there is positive cooperativity between the amino acid binding sites and between the tRNA^{Ser} binding sites. From the n values obtained from the Hill plots, cooperativity is also indicated, with a

minimum of two binding sites for each of the two substrates. In fluorescence studies [5], cooperativity has been previously indicated for the binding of tRNA^{Ser} to seryl-tRNA synthetase. In studies of protection against nuclease digestion [8] and in fluorescence measurements [5,7,39] two sites for tRNA^{Ser} were obtained. Unfortunately attempts to determine serine binding sites by equilibrium dialysis were unsuccessful, due to the low binding constant (which correlates with the relatively high " K_m ").

The kinetic data with ATP as variable substrate, are considerably more complex than those for the other two substrates. The curve form of the reciprocal plots shows several steps with an intermediate plateau; this curve form furthermore changes with the tRNA and amino acid concentrations. Although our data are more complex than the mathematical model [37], we favor as explanation of the shape of the curve that there are a series of consecutive negatively and positively interacting binding sites. The number of sites would appear to be greater than for the other substrates and variable depending upon concentration of other substrates. Probably some of the sites are unspecific and unproductive but in some way influential on the catalytic sites. In agreement with this model at least two binding sites for ATP were found in equilibrium dialysis and up to 10 in fluorescence measurements [39]. In this context it should be mentioned that ϵ ATP seems to follow normal Michaelis-Menten kinetics; possibly ϵ ATP fits into a catalytic site, but not into additional sites.

Several researchers have reported kinetic data for seryl-tRNA synthetase with tRNA^{Ser} as variable substrate [10,12,15,40,41]. Linear reciprocal plots were reported; this can be explained by technical reasons, such as the use of a narrower tRNA^{Ser} concentration range, which did not allow easy determination of the curvature of the plots. The K_m values for tRNA^{Ser} [10,12,15,40,41], however, are close to the " K_m " given in this paper. Makman and Cantoni [15] also reported K_m values for serine and ATP but did not specify the concentration ranges in which they were determined; again, the reported K_m values are comparable to the " K_m " values in this paper.

Studies on Phenylalanyl-tRNA Synthetase

In comparison to the seryl-tRNA synthetase system, the kinetics of Phe-tRNA^{Phe} formation are much less complex. There is one binding site for tRNA^{Phe} on the synthetase according to gradient centrifugation [42], fluorescence measurements [7] and nuclease protection studies [8]. Also for phenylalanine one binding site was found by equilibrium dialysis. In agreement with these results the evaluation of the kinetic data in Hill plot format indicates

one (or several independent and equivalent) binding site(s) for tRNA^{Phe} and phenylalanine.

Berther, Mayer and Dutler (unpublished results) report non-linear kinetic data at high concentrations of phenylalanine. The results of equilibrium dialysis experiments, plus our K_m value for phenylalanine (and their first K_m value) suggest that the deviation from linearity may be an induced effect at high substrate concentration.

For ATP linear reciprocal plots were obtained yielding $K_m = 0.8$ mM, a value comparable to those found by others [3,15]. H. Dutler pointed out to us that reciprocal plots for ATP were non-linear, approaching linearity only at low and high concentrations of the varied substrate. Our data (Fig. 3) point to a rather abrupt transition between two linear regions which could be attributed to several binding sites (> 2) for ATP with decreasing then increasing catalytic constants. A detailed investigation with curve fitting would be necessary to substantiate this detail.

Experiments with Aminoalkyl Adenylates

Aminoalkyl adenylates are valuable substances in the study of the mechanism of amino acid activation, since they interact with the synthetases in the presence of tRNA without leading to aminoacyl-tRNA formation [43,44]. The interactions can be observed in kinetic inhibition studies or in fluorescence experiments. We were interested in studying synthetase-tRNA interactions with synthetase molecules which had the other substrates, or better, non-reacting substrate analogs, bound to them. We therefore synthesized Phe-ol-pA and Ser-ol-pA, which were found to be competitive inhibitors of the cognate aminoacyl-tRNA synthetases with respect to amino acid. In both cases, the ratio K_m/K_i was of the same order of magnitude, indicating similarly strong inhibition. Competition with respect to ATP was not investigated. In studies of phenylalanyl-tRNA synthetase protection of tRNA^{Phe} against nuclease digestion, additional tRNA^{Phe} protection was found when Phe-ol-pA was present [8]. This additional protection disappeared when the tRNA was aminoacylated, while in the absence of Phe-ol-pA no difference in protection was detected between charged and uncharged tRNA. In initial fluorescence polarization studies no change in binding of modified tRNAs to the cognate synthetase was observed, when up to 1 mM Phe-ol-pA or Ser-ol-pA was added [7], although the degree of polarization was influenced by ATP.

ϵ A-Containing tRNAs and ϵ ATP

For the preparation of tRNA^{Ser} and tRNA^{Phe}, baker's yeast tRNAs were used, since these tRNAs lack the 3'-terminal AMP. As a result, in the

kinetic experiments modified tRNAs from baker's yeast were compared to unmodified tRNAs from brewer's yeast. Therefore, a comparison of the unmodified tRNAs from both sources is indicated.

tRNA^{Ser} from brewer's yeast consists of two major species [45], tRNA^{Ser}₁ and tRNA^{Ser}₂, which differ in only 3 nucleotides; tRNA^{Ser} from baker's yeast consists of only one major species, which is identical to tRNA^{Ser}₂ from brewer's yeast [46]. Since kinetic experiments with various separated tRNA^{Ser} species have shown the tRNAs to be very similar [12], the tRNA^{Ser} species from baker's and brewer's yeast seem to be equivalent for our purposes.

The structure of tRNA^{Phe} from baker's yeast has been elucidated [47] whereas no specific attempt has been made to sequence tRNA^{Phe} from brewer's yeast. Nevertheless, in rather extensive studies on fragments of brewer's yeast tRNA^{Phe}, no differences from the baker's yeast tRNA^{Phe} sequence were detected [9]. Thus, the phenylalanine-specific tRNAs from the two sources are not only equivalent for our purposes but are probably identical.

Bearing the foregoing in mind, one can conclude that the interchangeable use of the tRNAs from the two yeasts is justified. Moreover, the finding of only small differences in the kinetic properties of the ϵ A-containing and the unmodified tRNAs indicates that their modes of interaction with the synthetases are rather similar. This answers the originally posed question: there are no biochemical reasons against using the ϵ A-modified tRNAs in place of the unmodified ones in physicochemical studies. It seemed particularly interesting to use tRNA _{ϵ A} in fluorescence studies with aminoacyl-tRNA synthetase since the fluorescent label must be at the catalytic site of the enzyme during the aminoacylation process. Initial experiments along these lines show an increase in fluorescence intensity when seryl-tRNA synthetase is added to a solution of tRNA^{Ser} _{ϵ A} [7].

The finding that ϵ ATP functions in the aminoacylation reaction with phenylalanyl-tRNA and seryl-tRNA synthetases is in agreement with the report of Secrist *et al.* on tyrosyl-tRNA synthetase from pig pancreas [48]. The detailed analysis presented in this paper shows that, under conditions which are standard or optimal for ATP, ϵ ATP behaves rather differently. Although (and in some cases because) ϵ ATP is not equivalent to ATP, ϵ ATP constitutes an interesting probe in the study of synthetase interactions.

We thank R. Hirsch for advice on the preparation of the aminoacyl-tRNA synthetases, W. Schäfer for the mass spectra, and H. Dutler for informing us of his results prior to publication. We are indebted to E. Wünsch and F. Drew who provided the starting material for and helpful discussion during the synthesis of Ser-ol-pA. Equilibrium dialysis in the presence of Phe-ol-pA was suggested by J. P. Waller, who critically read the manuscript as

S. Blanquet and H. Witzel. The expert assistance of S. Notz in the kinetic experiments is gratefully acknowledged. H. Hertz wishes to thank the *Alexander von Humboldt Stiftung* for a fellowship. The work was supported by *Deutsche Forschungsgemeinschaft*, SFB 51.

Note Added in Proof (6.7.1973). The experiments in this paper were performed with a phenylalanyl-tRNA synthetase preparation having the properties described by Fasiolo *et al.* [3]. Recently synthetase preparations were obtained (R. Hirsch, unpublished) the activity of which (in nmoles Phe-min⁻¹) approaches 2000 units/A₂₈₀ unit under assay conditions similar to those of Fasiolo *et al.* [3] and 4000 units/A₂₈₀ unit under the conditions of J. Schmidt *et al.* (1971) *Biochemistry*, 10, 3264–3268. In equilibrium dialysis experiment the affinity for phenylalanine was somewhat higher; as before, one binding site per synthetase molecule was found by dialysis in the presence of 0, 0.5, and 1.0 mole Phe-ol-pA per mole of synthetase. In nuclease protection studies (W. Hörz, personal communication) the 1:1 stoichiometry of the tRNA-synthetase complexes remained as previously reported [8]. The two groups of experiments indicate that the previously used phenylalanyl-tRNA synthetase preparations contained inactive but ligand binding enzyme molecules.

The equilibrium dialysis experiments with seryl-tRNA synthetase were continued under the above described conditions using "Isis 3069" membranes from Société des Usines Chimiques Rhône-Poulenc, Paris (obtained through the courtesy of A. Richard). Two binding sites for ATP were observed, with binding constants of 2×10^4 l. mol⁻¹ and 0.5×10^4 l. mol⁻¹, respectively. This compares to the more than two binding sites for ATP, under aminoacylation conditions, which were indicated by the kinetic analysis.

REFERENCES

- Loftfield, R. B. (1972) *Progr. Nucleic Acid Res. Mol. Biol.* 12, 87–128.
- Mehler, A. H. & Chakraborty, K. (1971) *Adv. Enzymol.* 35, 443–501.
- Fasiolo, F., Befort, N., Boulanger, Y. & Ebel, J. P. (1970) *Biochim. Biophys. Acta*, 217, 305–318.
- Rigler, R., Cronvall, E., Hirsch, R., Pachmann, U. & Zachau, H. G. (1970) *FEBS Lett.* 11, 320–323.
- Rigler, R., Cronvall, E., Ehrenberg, M., Pachmann, U., Hirsch, R. & Zachau, H. G. (1971) *FEBS Lett.* 18, 193–198.
- Heider, H., Gottschalk, E. & Cramer, F. (1971) *Eur. J. Biochem.* 20, 144–152.
- Pachmann, U., Cronvall, E., Rigler, R., Hirsch, R., Wintermeyer, W. & Zachau, H. G., (1973) *Eur. J. Biochem.*, in press.
- Hörz, W. & Zachau, H. G. (1973) *Eur. J. Biochem.* 32, 1–14.
- Thiebe, R., Harbers, K. & Zachau, H. G. (1972) *Eur. J. Biochem.* 26, 144–152.
- Fittler, F. & Zachau, H. G. (1973) *Arch. Biochem. Biophys.* 155, 368–380.
- Barrio, J. R., Secrist, J. A., III & Leonard, N. J. (1972) *Biochem. Biophys. Res. Commun.* 46, 597–604.
- Kruppa, J. & Zachau, H. G. (1972) *Biochim. Biophys. Acta*, 277, 499–512.
- Fittler, F., Kruppa, J. & Zachau, H. G. (1972) *Biochim. Biophys. Acta*, 277, 513–522.
- Zachau, H. G. (1970) in *Methoden der enzymatischen Analyse*, 2. Aufl., Band II (Bergmeyer, H. U., ed.) pp. 1828–1834, Verlag Chemie, Weinheim/Bergstr.
- Makman, M. H. & Cantoni, G. L. (1965) *Biochemistry*, 4, 1434–1442.
- Overath, H., Fittler, F., Harbers, K., Thiebe, R. & Zachau, H. G. (1970) *FEBS Lett.* 11, 289–294.
- Sandrin, E. & Boissonnas, R. A. (1966) *Helv. Chim. Acta*, 49, 76–82.
- Wünsch, E. & Jentsch, J. (1964) *Chem. Ber.* 97, 2490–2496.
- Spangenberg, R., Thamm, P. & Wünsch, E. (1971) *Hoppe-Seyler's Z. Physiol. Chem.* 352, 655–656.
- Graßmann, W., Hörmann, H. & Endres, H. (1955) *Chem. Ber.* 88, 102–117.
- Rammler, D. H. & Khorana, H. G. (1962) *J. Am. Chem. Soc.* 84, 3112–3122.
- Gordon, H. T., Thornburg, W. & Werum, L. N. (1956) *Anal. Chem.* 28, 849–855.
- Razzell, W. E. (1963) *Methods Enzymol.* 6, 236–258.
- Lineweaver, H. & Burk, D. (1934) *J. Am. Chem. Soc.* 56, 658–666.
- Secrist, J. A., III, Barrio, J. R., Leonard, N. J. & Weber, G. (1972) *Biochemistry*, 11, 3499–3506.
- Monod, J., Changeux, J. P. & Jacob, F. (1963) *J. Mol. Biol.* 6, 306–329.
- Atkinson, D. E., Hathaway, J. A. & Smith, E. C. (1965) *J. Biol. Chem.* 240, 2682–2690.
- Reference deleted.
- Aebi, H. (1964) in *Biochemisches Taschenbuch*, 2. Aufl., 2. Teil (Rauen, H. M., ed.) pp. 159–192, Springer-Verlag, Berlin, Göttingen, Heidelberg.
- Scatchard, G. (1949) *Ann. N.Y. Acad. Sci.* 51, 660–672.
- Myers, G., Blank, H. U. & Söll, D. (1971) *J. Biol. Chem.* 246, 4955–4964.
- Papas, T. S. & Peterkofsky, A. (1972) *Biochemistry*, 11, 4602–4608.
- Makman, M. H. & Cantoni, G. L. (1966) *Biochemistry*, 5, 2246–2254.
- Cleland, W. W. (1970) in *The Enzymes*, 3rd ed., vol. II, (Boyer, P. D., ed.) pp. 1–65, Academic Press, New York.
- Reiner, J. M. (1959) *Behavior of Enzyme Systems*, pp. 1–73, Burgess Publishing Company, Minneapolis, Minn.
- Engel, P. C. & Ferdinand, W. (1973) *Biochem. J.* 131, 97–105.
- Teipel, J. & Koshland, D. E., Jr (1969) *Biochemistry*, 8, 4656–4663.
- Changeux, J. P. (1963) *Cold Spring Harbor Symp. Quant. Biol.* 28, 497–504.
- Engel, G., Heider, H., Maelicke, A., v. d. Haar, F. & Cramer, F. (1972) *Eur. J. Biochem.* 29, 257–262.
- Igo-Kemenes, T. & Zachau, H. G. (1969) *Eur. J. Biochem.* 10, 549–556.
- Hirsch, R. & Zachau, H. G. (1970) *Hoppe-Seyler's Z. Physiol. Chem.* 351, 563–566.
- Befort, N., Fasiolo, F., Bollack, C. & Ebel, J. P. (1970) *Biochim. Biophys. Acta*, 217, 319–331.
- Cassio, D., Lemoine, F., Waller, J. P., Sandrin, E. & Boissonnas, R. A. (1967) *Biochemistry*, 6, 827–835.
- Blanquet, S., Fayat, G., Waller, J. P. & Iwatsubo, M. (1972) *Eur. J. Biochem.* 24, 461–469.
- Zachau, H. G., Dütting, D. & Feldmann, H. (1966) *Hoppe-Seyler's Z. Physiol. Chem.* 347, 212–235.
- Neelon, F. A., Molinaro, M., Ishikura, H., Sheiner, L. B. & Cantoni, G. L. (1967) *J. Biol. Chem.* 242, 4515–4522.
- RajBhandary, U. L., Chang, S. H., Stuart, A., Faulkner, R. D., Hoskinson, R. M. & Khorana, H. G. (1967) *Proc. Natl. Acad. Sci. U. S. A.* 57, 751–758.
- Secrist, J. A., III, Barrio, J. R. & Leonard, N. J. (1972) *Science (Wash. D. C.)* 175, 646–647.

H. S. Hertz and H. G. Zachau, Institut für Physiologische Chemie und Physikalische Biochemie der Universität, D-8000 München 15, Goethestraße 33, Federal Republic of Germany

Fluorescent Affinity Labeling of Initiation Site on Ribonucleic Acid Polymerase of *Escherichia coli*[†]

Felicia Y.-H. Wu and Cheng-Wen Wu*,[‡]

ABSTRACT: A fluorescent analog of 6-methylthioinosinedicarboxaldehyde (MMPR-OP) has been synthesized in which the methyl group is replaced by *N*-(acetyl aminoethyl)-1-naphthylamine-5-sulfonate. This fluorescent nucleotide analog (AMPR-OP) is a much more potent inhibitor of DNA dependent RNA polymerase of *Escherichia coli* than MMPR-OP. The concentration of AMPR-OP required to inhibit 50% of RNA polymerase activity is 7×10^{-6} M as compared to 5×10^{-4} M for MMPR-OP. The noncompetitive inhibition of AMPR-OP with respect to nucleoside triphosphate suggests that AMPR-OP binds to a site on the enzyme involved in the initiation of RNA chains. The inhibition of DNA dependent [³²P]PP_i exchange reaction by low concentrations of AMPR-OP further support the contention that this compound primarily inhibits the initiation of RNA chains. When RNA polymerase was incubated with excess AMPR-OP followed by

NaBH₄ reduction, the dye was stoichiometrically bound to the enzyme. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the denatured, labeled enzyme indicates that AMPR-OP is covalently attached to the β subunit of the enzyme. Although the labeled enzyme is essentially inactive, fluorescence studies show that it still retains the ability to bind DNA template and nucleoside triphosphates. The binding of nucleoside triphosphates is presumably to the second nucleotide site (the polymerization site) on the enzyme and its specificity is dependent on the template. Furthermore, upon binding of the template and nucleoside triphosphates, the enzyme undergoes conformational changes. Energy transfer measurements indicate that the initiation site and rifampicin binding site are at least 37 Å apart. Thus the inhibitory effects of rifampicin on initiation of RNA chains is indirectly mediated through enzyme molecule.

Fluorescent probes have been used to provide insight into the structure, interactions, and dynamics of macromolecules. By systematically labeling multiple active sites of DNA dependent RNA polymerase of *Escherichia coli* with various fluorescent probes, we have examined the molecular mechanism of gene transcription (Wu and Wu, 1973a-c).

Recently, Spoor *et al.* (1970) have shown that the periodate oxidation product of 6-methylmercaptapurine ribonucleoside (MMPR-OP)¹ inhibited *Escherichia coli* RNA polymerase by covalently binding to the initiation site (the first NTP site, Wu and Goldthwait, 1969; or the product terminus site, Krakow and Fronk, 1969) on the enzyme. The binding site has been shown to be an ϵ -amino group of a lysine residue in the β subunit of RNA polymerase.

We report here the affinity labeling of *Escherichia coli* RNA polymerase with a fluorescent analog of MMPR-OP in which the methyl group is replaced by *N*-(acetyl aminoethyl)-1-naphthylamine-5-sulfonate (AMPR-OP, Figure 1). Like MMPR-OP, AMPR-OP also binds to the initiation site on the β subunit. Although the AMPR-OP-labeled enzyme is cat-

alytically inactive, it still interacts with DNA template and nucleoside triphosphates. A model of the active sites of RNA polymerase consistent with the fluorescence spectroscopic results is discussed. Furthermore, energy transfer measurements have been carried out to elucidate spatial and functional relationships between the initiation site on the enzyme and the binding site of rifampicin, which is a specific inhibitor of RNA chain initiation.

Materials and Methods

Materials. Unlabeled ribonucleoside triphosphates were purchased from P-L Biochemicals. ³H-labeled ribonucleoside triphosphates and ³²P-labeled sodium pyrophosphate were obtained from New England Nuclear Corp. Poly[d-(A-T)] and calf thymus DNA were products of Miles Laboratories, Inc., and Worthington Biochemical Corp., respectively. 6-Mercaptopurine ribonucleoside (6-MPR) was obtained from Cyclochemicals and sodium periodate from Fisher Scientific Co. Tricine, sodium borohydride, and dithiothreitol were purchased from Sigma. Unlabeled and [¹⁴C]-rifampicin were gifts of Drs. R. White and L. Sylvestri of Gruppo-Lepetit Laboratories. *N*-(Iodoacetyl aminoethyl)-1-naphthylamine-5-sulfonate (1,5 I-AENS) was synthesized by the method of Hudson and Weber (1973). Silica gel plates for thin-layer chromatography were obtained from Eastman Organic Chemicals. Sephadex G-75 was the product of Pharmacia Fine Chemicals, Inc.

RNA Polymerase. RNA polymerase was purified from *E. coli* as described by Wu and Wu (1973c). The enzyme was 98% pure, and contained all subunits (α , β , β' , and σ) as shown by sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis.

RNA Polymerase Activity Assay. Enzyme activities of the labeled and unlabeled RNA polymerase were assayed by the

[†] From the Division of Biological Sciences, Department of Biophysics, Albert Einstein College of Medicine, Bronx, New York 10461. Received January 21, 1974. This work was supported in part by research grants from the National Institutes of Health (GM 19062) and the American Cancer Society (BC-94).

[‡] Research Career Development Awardee of the National Institutes of Health.

¹ Abbreviations used are: NTP, nucleoside triphosphate; PP_i, inorganic pyrophosphate; 6-MPR, 6-mercaptopurine ribonucleoside; MMPR, 6-methylmercaptapurine ribonucleoside; MMPR-OP, oxidation product of MMPR, i.e., methylthioinosinedicarboxaldehyde; 1,5 I-AENS, *N*-(iodoacetyl aminoethyl)-1-naphthylamine-5-sulfonate; AENS, *N*-(acetyl aminoethyl)-1-naphthylamine-5-sulfonate group; AMPR, *N*-(acetyl aminoethyl)-1-naphthylamine-5-sulfonate derivative of 6-MPR, 5-[[2-[[[(9- β -D-ribofuranosyl-9-H-purin-6-yl)thio]acetyl]amino]ethyl]amino]-1-naphthalenesulfonate; AMPR-OP, oxidation product of AMPR.

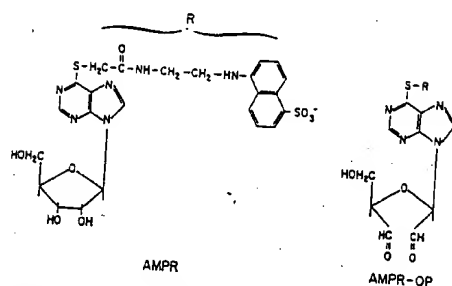


FIGURE 1: Structures of AMPR and AMPR-OP.

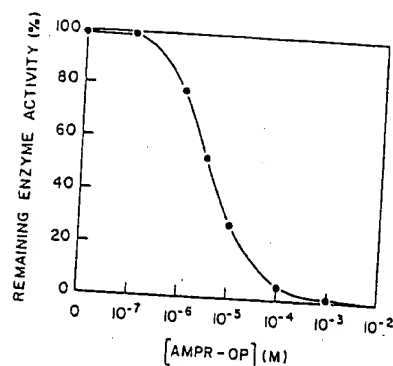


FIGURE 2: The dependence of RNA synthesis on the concentration of AMPR-OP. The per cent of enzyme activity remaining after exposure to AMPR-OP is plotted against the concentration of AMPR-OP added. The reaction mixture and conditions of the activity assay were as described in the Materials and Methods section.

bound to the gel electro- indicates that bunit of the ally inactive, bility to bind ie binding of econd nucleo- zyme and its ermore, upon osphates, the ergy transfer and rifampicin ibitory effects is indirectly

template and e sites of RNA e spectroscopic e measurements i functional re- enzyme and the fic inhibitor of

hosphates were eled ribonucleo- i pyrophosphate r Corp. Poly(d- ts of Miles Lab- al Corp., respec- -MPR) was ob- periodate from- dride, and dithio- beled and [¹⁴C]- id L. Sylvestri- etylaminoethyl)- i as synthesized by ilica gel plates for d from Eastman ; the product of

s purified from E. The enzyme was , β, β', and σ) as 'lamide gel electro-

ne activities of the vere assayed by the

incorporation of ³H-labeled ribonucleoside monophosphate into acid-insoluble material using the procedure described previously (Wu and Wu, 1973c). The enzyme (5 μg) was pre-incubated at 37° for 15 min with 80 mM Tris-HCl (pH 7.8), 10 mM MgCl₂, and various concentrations of nucleoside analog inhibitor or water. After preincubation the reaction mixtures were cooled to 4° and to these were added 0.12 mM calf thymus DNA, 0.4 mM each of ATP, CTP, UTP, and GTP (one labeled with ³H, 8 × 10³ cpm/nmol), 1.6 mM Na₂HPO₄, and 4 mM β-mercaptoethanol. When poly[d(A-T)] was used as a template, GTP and CTP were omitted and 0.2 M KCl was added to the reaction mixture. The complete system (0.25 ml) was further incubated at 37° for 20 min and reactions were terminated by cooling the mixtures to 4°, adding 0.1 ml of 0.1 M sodium pyrophosphate and 5 ml of 5% trichloroacetic acid. The acid-insoluble precipitates were collected on a glass fiber filter (Whatman GF/C, 2.4 cm) and washed with cold 1% trichloroacetic acid and 95% ethanol. The filter was dried and the radioactivity was measured with a liquid scintillation spectrophotometer.

Synthesis of AMPR and AMPR-OP. Equimolar quantities of 1,5 I-AENS (22 mg) and 6-MPR (14 mg) in 0.1 N NaOH (1 ml) were reacted in the dark at room temperature for 3 hr and then at 4° overnight. Quantitative yield of the addition product, AMPR, was obtained after evaporation to dryness and recrystallization from acetone. AMPR (28 mg) was then oxidized with an equimolar amount of sodium periodate (10.7 mg) in H₂O (3 ml) in the dark at room temperature for 5 hr. After addition of 95% ethanol, the white precipitate was filtered. The alcohol solution was evaporated to dryness and brown crystals of AMPR-OP were obtained in 50% yield after recrystallization from ethanol-water. Thin-layer chromatography of the reactants and products on silica gel in 5% Na₂HPO₄ solvent system gave a single spot having R_F values of 0.50, 0.59, 0.65, and 0.80 for 1,5 I-AENS, AMPR-OP, AMPR, and 6-MPR, respectively. The molar absorption coefficients of AMPR-OP in aqueous solution (pH 7) are 4.7 × 10³ M⁻¹ cm⁻¹ at 355 nm (shoulder), 3.5 × 10⁴ M⁻¹ cm⁻¹ at 285 nm, and 2.7 × 10⁴ M⁻¹ cm⁻¹ at 265 nm (shoulder). The fluorescent emission maximum of AMPR-OP is at 485 nm.

DNA Dependent [³²P]PP_i Exchange Reaction. The DNA dependent [³²P]PP_i exchange reaction catalyzed by RNA polymerase was measured as described by Krakow and Fronck (1969).

Labeling of RNA Polymerase with AMPR-OP. In labeling experiments, 1.2 mg of enzyme was first dialyzed overnight against 0.1 M NaHCO₃ or Tricine buffer (pH 7.9) containing 8 mM MgCl₂, 0.2 M KCl, and 0.1 mM dithiothreitol to remove Tris normally present in the enzyme storage buffer. The enzyme was then incubated for 45 min at 37° with a 10- to 300-fold molar excess of AMPR-OP. After incubation, the reaction mixture was cooled to 4° and 2-20 mg of NaBH₄ in 1 ml of NaHCO₃ buffer was added. Reduction of the Schiff base to

a stable covalent bond was allowed to occur for 16 hr at 4°. The entire reaction mixture was then passed through a Sephadex G-75 column (1 × 14 cm) to remove unreacted AMPR-OP and NaBH₄. The labeled enzyme was eluted with 50 mM Tris-HCl buffer (pH 8) containing 0.5 M KCl, 0.1 mM EDTA, and 0.1 mM dithiothreitol. This step was followed by extensive dialysis against the same buffer.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis Analysis. Electrophoresis on polyacrylamide gel was performed according to the method described by Weber and Osborn (1969). The AMPR-OP-bound enzyme was denatured with 3% sodium dodecyl sulfate or 7 M urea. Approximately 50 μg of denatured protein was layered on 7.5% polyacrylamide gels. Gels were run for 4-5 hr at 8 mA/tube. The gels were stained with Coomassie Brilliant Blue (0.2% methanol-acetic acid-H₂O, 5:1:5) overnight; then destained with the same solvent mixture as above until the gels yielded clear, visible bands.

Spectroscopic measurements were carried out with a Cary 118C recording spectrophotometer in a 1-cm light-path quartz cell. All spectroscopic measurements were carried out at 22 ± 0.1°.

Fluorescence excitation and emission spectra were recorded in a Hitachi-Perkin-Elmer fluorescence spectrophotometer, Model MPF-3, equipped with a corrected spectra accessory. The solutions used for fluorescence studies had absorbances of less than 0.05 at the excitation wavelength to obviate inner filter effect.

Quantum yield ϕ of a sample was calculated from absorbance (A) and the area enclosed by the corrected emission spectrum using the relationship (Parker and Rees, 1960)

$$\phi_S = \phi_R \frac{(1-10^{-A_R})}{(1-10^{-A_S})} \frac{(\text{area})_S}{(\text{area})_R} \frac{n_R^2}{n_S^2} \quad (1)$$

where n is the refractive index of the solvent, and S and R refer to sample and reference, respectively. 5-Anilino-naphthalene-1-sulfonate in ethanol was used as a reference of quantum yield 0.37 (Stryer, 1965). The areas of the corrected emission spectra were obtained by planimetry.

Results

Inhibition of RNA Polymerase Activities by AMPR-OP. Figure 2 shows the effect of various concentrations of AMPR-OP on the DNA dependent RNA synthesis catalyzed by *Escherichia coli* polymerase. Virtually no inhibition occurred up to 10⁻⁷ M, while complete inhibition was achieved at about

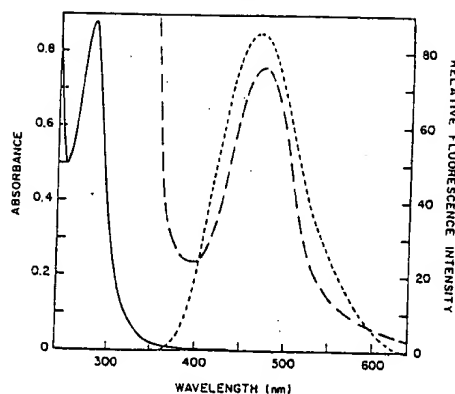


FIGURE 3: Absorption and corrected fluorescence emission spectra of AMPR-OP labeled polymerase and the overlap with the absorption spectrum of rifampicin: (—) absorption spectrum of 1.5×10^{-6} M labeled RNA polymerase in 0.5 M KCl–0.05 M Tris–HCl (pH 8)–0.1 mM EDTA–0.1 mM dithiothreitol; (-----) corrected fluorescence emission spectrum of 5×10^{-7} M labeled RNA polymerase in the same buffer, the excitation wavelength was at 330 nm; (---) absorption spectrum of 7.2×10^{-6} M rifampicin in the same buffer.

10^{-4} M. The concentration of AMPR-OP required for 50% inhibition was about 7×10^{-6} M, which is 100 times smaller than that of MMPR-OP for the same extent of inhibition.

The effect of nucleotide concentration on AMPR-OP inhibition was studied using poly[d(A-T)] as template. At a saturating concentration of UTP (0.4 mM), variation of the concentration of the alternate nucleoside triphosphate, ATP, yielded a linear double reciprocal plot. The results showed that the V_{max} decreased in the presence of AMPR-OP (the values of V_{max} are 1.0, 0.83, and 0.43 nmol/min at AMPR-OP concentrations of 0, 4×10^{-6} , and 1×10^{-5} M), while the apparent K_m remained unchanged (7×10^{-6} M), suggesting that AMPR-OP is a noncompetitive inhibitor with respect to ATP binding to RNA polymerase. The K_i value obtained for AMPR-OP was 7.4×10^{-6} M.

The type of inhibition produced by AMPR-OP is the same as that by MMPR-OP (Spoor *et al.*, 1970). This suggests that like MMPR-OP, AMPR-OP might bind to the initiation site on the enzyme. (If it binds to the polymerization site, a competitive type of inhibition would be expected.) To further support this contention, the effect of AMPR-OP on the poly[d(A-T)]-dependent [32 P]PP_i exchange reaction was examined. As shown in Table I, at 0.1 mM AMPR-OP the incorporation of [32 P]PP_i was almost completely inhibited. Thus, AMPR-OP primarily inhibits the initiation of RNA chains in the RNA polymerase reaction.

Affinity Labeling of RNA Polymerase with AMPR-OP. When the holoenzyme of RNA polymerase was incubated with excess AMPR-OP and then reduced by NaBH₄, AMPR-OP was bound to the enzyme in about 1:1 molar ratio. Prolonged incubation with a large excess of the dye did not significantly alter this stoichiometry. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the labeled enzyme (Weber and Osborn, 1969) showed a fluorescent band corresponding to the β subunit; bands corresponding to other subunits were nonfluorescent. This indicated that AMPR-OP was covalently bound to the β subunit of RNA polymerase.

The labeled RNA polymerase was essentially inactive (<1%) in DNA dependent polymerization and PP_i exchange reactions. The absorption and fluorescence emission spectra of the labeled enzyme are shown in Figure 3. The absorption maximum for the modified protein was at 280 nm with a tail at 300–350 nm due to the bound dye. The fluorescence ex-

TABLE I: Effect of AMPR-OP on Poly[d(A-T)] Dependent [32 P]PP_i-Exchange Reaction.^a

AMPR-OP Added (M)	[32 P]PP _i Incorporation (nmol)	Inhibition (%)
0	37.4	0
1×10^{-5}	9.9	74
1×10^{-4}	3.0	92

^a The incorporation of [32 P]PP_i into nucleoside triphosphates was measured by adsorption to activated charcoal (Krakow and Fronk, 1969). The complete system (0.25 ml) contained 80 mM Tris–HCl (pH 7.8), 40 mM β -mercaptoethanol, 4 mM MgCl₂, 0.4 mM UTP, 1 mM sodium [32 P]-pyrophosphate (1.4×10^4 cpm/nmol), 0.1 A₂₆₀ unit of poly[d(A-T)], and 5 μ g of RNA polymerase. The incubation was for 10 min at 37° and reactions were stopped by addition of 0.2 ml of 0.1 M EDTA (pH 6.0), and 0.1 ml of 0.1 M sodium pyrophosphate (pH 6.0), followed by addition of 0.5 ml of a 10% suspension of acid-washed, activated charcoal in 0.01 M sodium pyrophosphate (pH 6.0). After mixing, 3 ml of 0.01 M sodium pyrophosphate was added and the mixture was filtered through glass-fiber filters. The filters were washed with 40 ml of 0.01 M sodium pyrophosphate, dried, and counted.

citation and emission maxima were at 335 (not shown) and 470 nm, respectively.

Interaction of the Labeled Enzyme with DNA and Nucleotides. When calf thymus DNA (100 μ g) or poly[d(A-T)] (same amount) was added to a solution containing 10^{-8} M labeled polymerase, 10 mM MgCl₂, 0.2 M KCl, 0.1 mM dithiothreitol, and 0.05 M Tris–HCl (pH 7.8) there was a 20–30% increase in the fluorescence intensity of the labeled enzyme and a small blue shift (3 nm) of the emission maximum. In the absence of DNA, addition of 0.4 mM of a single nucleoside triphosphate (ATP, GTP, UTP, or CTP) to 10^{-8} M labeled enzyme in the same buffer brought about a 5-nm blue shift of the emission maximum and a small (about 5–7%) enhancement of the fluorescence intensity. These observations occurred with any one of the four nucleoside triphosphates, and the effect of more than two nucleoside triphosphates was less than additive. In the presence of calf thymus DNA, however, the observed fluorescence enhancement was much larger, about 20% increase by each nucleoside triphosphate. If poly[d(A-T)] was present instead of calf thymus DNA, the situation was quite different. Addition of ATP (0.4 mM) did not significantly alter the fluorescent properties of the probe, whereas addition of UTP (0.4 mM) markedly enhanced the fluorescence intensity (30%). Addition of AMP, UMP, ADP, or UDP has no effect on the fluorescent properties of the labeled enzyme in the presence of either calf thymus DNA or poly[d(A-T)].

Energy Transfer from the Initiation Site to the Rifampicin Binding Site on RNA Polymerase. Rifampicin, a specific inhibitor of RNA chain initiation, has been shown to bind to a single site on RNA polymerase (Zillig *et al.*, 1970). The modification of RNA polymerase by AMPR-OP did not significantly alter its ability to bind rifampicin. By use of a gel filtration technique (Yarbrough and Wu, 1974), we found that the labeled enzyme bound 0.5 mol of [3 H]rifampicin per mole of enzyme while the unlabeled enzyme bound 0.6 mol of [3 H]rifampicin per mole of enzyme under the same experimental conditions. Thus energy transfer measurements were carried out to estimate the distance between the rifampicin

Dependent

ition (%)

0

74

92

de triphos-
d charcoal
n (0.25 ml)
3-mercapto-
dium [³²P]-
t of polyd-
ion was for
lition of 0.2
dium pyro-
nl of a 10%
in 0.01 M
ml of 0.01 M
was filtered
l with 40 ml
ed.

shown) and

'Nucleotides
A-T)] (same
-8 M labeled
ithiothreitol
% increase in
and a small
the absence
side triphos-
ealed enzyme
shift of the
enhancement
ns occurred
ates, and the
ates was less
JA, however,
much larger,
hosphate. If
is DNA, the
(0.4 mM) did
of the probe,
enhanced the
UMP, ADP,
erties of the
thymus DNA

he Rifampicin
in, a specific
own to bind
L., 1970). The
-OP did not
by use of a gel
we found that
icin per mole
d 0.6 mol of
same experi-
ments were
he rifampicin

binding site and the initiation site on RNA polymerase because rifampicin, which possesses an absorption maximum at 470 nm, is an ideal energy acceptor of the emission of AMPR-OP (Figure 3).

In Förster's theory of dipole-dipole energy transfer (Förster, 1947), the transfer efficiency (E) is related to the distance (r) between the donor and acceptor by

$$E = r^{-6}/(r^{-6} + R_0^{-6}) \quad (2)$$

R_0 , the distance (in Å) at which the transfer efficiency is 50%, is given by

$$R_0 = (JK^2Q_0n^{-4})^{1/6}(9.79 \times 10^3) \quad (3)$$

where K^2 is the orientation factor for dipole-dipole transfer, Q_0 is the quantum yield of the donor in the absence of transfer, n is the refractive index of the medium, and J is the spectral overlap integral calculated from the emission spectrum of the donor and the absorption spectrum of the acceptor. Q_0 , the quantum yield of the enzyme-bound AMPR-OP, was determined to be 0.05 with 8-anilino-1-naphthalenesulfonate in ethanol as a reference of quantum yield 0.37 (Stryer, 1965). The value of J was $3.3 \times 10^{-14} \text{ cm}^2 \text{ M}^{-1}$ as estimated from the corrected fluorescence emission spectrum of the enzyme-bound AMPR-OP and the absorption spectrum of the enzyme-bound rifampicin (Figure 3) using the equation

$$J = \frac{\int F(\bar{\nu})\epsilon(\bar{\nu})\bar{\nu}^4 d\bar{\nu}}{\int F(\bar{\nu})d\bar{\nu}} \quad (4)$$

where $F(\bar{\nu})$ is the fluorescence intensity of the donor at wave number $\bar{\nu}$, and $\epsilon(\bar{\nu})$ is the extinction coefficient of the energy acceptor at that wave number. Though n cannot be measured directly, there is little uncertainty as to its value, which we assume to be 1.4. The problem arises in assuming a value to the orientation factor K^2 . If the relative orientation of donor-acceptor pairs is completely randomized during the excited state lifetime, K^2 equals $2/3$. Although the orientation of the energy acceptor is not known, nanosecond emission anisotropy measurements show that the energy donor used in this study has local rotational mobility (C.-W. Wu and F. Y.-H. Wu, to be published). The rapid decrease in emission anisotropy (from 0.32 to 0.08) within 7 nsec indicates that the energy donor rotates over an angle of the order of 60° . (The excited state lifetime of the enzyme-bound AMPR-OP was 15 nsec.) The value of R_0 was calculated to be 25 Å for the AMPR-OP and rifampicin pair on RNA polymerase using the experimentally observed values of J and Q_0 and assuming that $n = 1.4$ and $K^2 = 2/3$. If an energy donor has complete rotational freedom and an energy acceptor is fixed, K^2 can range from $1/2$ to $4/3$. Since R_0 is proportional to $1/6$ power of K^2 , the factor of 2 variations in K^2 will result in 12% error in the value of R_0 .

The transfer efficiency, E , was determined from quantum yields of the donor in the presence and absence of energy acceptor (Q and Q_0 , respectively). When rifampicin (6 μM)

$$E = 1 - (Q/Q_0) \quad (5)$$

was added to a solution of the labeled enzyme, a 10% decrease in quantum yield was observed. Thus an apparent distance between the initiation site and the rifampicin binding site on RNA polymerase was calculated to be 37 Å according to eq 2.

Discussion

Spoor *et al.* (1970) have shown that MMPR-OP is a potent inhibitor of *Escherichia coli* DNA dependent RNA polym-

erase. By substituting the methyl group of MMPR-OP with a fluorescent chromophore, *N*-(acetylaminooethyl)-1-naphthylamine-5-sulfonate (AENS), we found that the new pseudo-substrate, AMPR-OP, was a much more potent inhibitor of RNA polymerase. The K_i of AMPR-OP ($7.4 \times 10^{-6} \text{ M}$) was two orders of magnitude smaller than that of MMPR-OP ($5.1 \times 10^{-4} \text{ M}$). Although AMPR-OP is a larger molecule, the result indicates that the fluorescent chromophore somehow stabilizes the inhibitor-enzyme complex.

Since noncompetitive inhibition was observed for MMPR-OP with respect to polymerization of nucleoside triphosphates (Spoor *et al.*, 1970), it was proposed that the inhibitor bound at the initiation site, a nucleotide binding site on the enzyme other than the polymerization (elongation) site. Similar non-competitive kinetics was observed for AMPR-OP, suggesting that AMPR-OP may also bind to the initiation site.

The binding site of MMPR-OP has been shown to consist of an ϵ -amino group of a lysine residue in the β subunit of RNA polymerase (Nixon *et al.*, 1972). If AMPR-OP and MMPR-OP were bound to the same site, AMPR-OP should also form a Schiff base with the ϵ -amino group of the lysine. In fact, after sodium borohydride reduction, a stable covalent linkage between AMPR-OP and the β subunit of RNA polymerase was obtained as shown by the sodium dodecyl sulfate polyacrylamide gel electrophoresis of the labeled enzyme.

The labeled polymerase was essentially inactive in catalyzing RNA synthesis and the DNA dependent PP_i exchange reaction. However, the altered enzyme still could interact with DNA template and nucleoside triphosphates as demonstrated by the enhancement of fluorescence intensity and the blue shifts of the emission maximum. Since the fluorescent probe used here is sensitive to the environment (Hudson and Weber, 1973), these observations indicate that the surrounding of the AMPR-OP binding site has become less polar upon binding to DNA or nucleoside triphosphate, *i.e.*, a template- or substrate-induced conformational change of the enzyme has taken place. (No direct interaction between AMPR-OP and DNA or NTP could be detected by fluorescence measurements.) In addition, the emission maximum of free AMPR-OP is at 485 nm and that of the bound AMPR-OP is at 470 nm. This implies that the environment of the initiation is slightly more hydrophobic than that of the aqueous media.

Wu and Goldthwait (1969) have demonstrated two nucleoside triphosphate binding sites on *Escherichia coli* RNA polymerase (in the absence of DNA): a weak binding site ($K_s = 1.5 \times 10^{-4} \text{ M}$) with preferential affinity for purine nucleotides, and a strong binding site ($K_s = 1.5 \times 10^{-5} \text{ M}$) for all four nucleoside triphosphates. Kinetic analysis (Anthony *et al.*, 1969) has suggested that the weak binding site is the initiation site and the strong binding site, the polymerization site. In this paper, we have shown that the affinity-label very probably binds to the initiation site of the enzyme (although it is not really proved). If AMPR-OP were bound to the initiation site by the affinity labeling as suggested above, then nucleoside triphosphate must interact with the other binding site, the polymerization site on the labeled enzyme. This is consistent with the observation that in the absence of DNA, all four nucleoside triphosphates produce similar fluorescence changes of the labeled enzyme.

The most interesting findings are the interactions between nucleoside triphosphates and the labeled enzyme in the presence of DNA. The further increase in fluorescence intensity of the labeled enzyme by binding nucleoside triphosphates in the presence of calf thymus DNA, as compared to

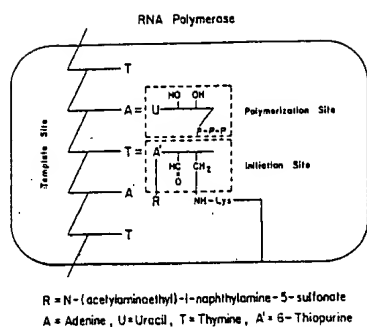


FIGURE 4: Model for active sites on RNA polymerase of *Escherichia coli*.

that in the absence of DNA, may reflect some structural alteration of the polymerization site (on the enzyme) by the template. This is best demonstrated when d(A-T) copolymer was used as the template. As shown in the model presented in Figure 4, AMPR-OP is covalently attached to a lysine residue at or near the initiation site. When d(A-T) copolymer occupies the template site on the enzyme, the purine moiety of AMPR-OP may form hydrogen bonds with a thymine base of the template. The specificity of the polymerization site is then governed by the adjacent adenine base of the template due to base complementation (A-U hydrogen bonding), or alternatively, the adenine moiety of the template may induce a conformational change of the enzyme so that the polymerization site binds UTP preferentially. Our observation that in the presence of poly[d(A-T)] UTP but not ATP markedly enhanced the fluorescence of the labeled enzyme can be readily explained by this model.

Rifampicin is a known inhibitor of *Escherichia coli* RNA polymerase (Hartmann *et al.*, 1967). It binds to a single site on the enzyme and genetic evidence suggests that the rifampicin binding site is on the β subunit of the enzyme (Rabussay and Zillig, 1969; Zillig *et al.*, 1970; Heil and Zillig, 1970). Since rifampicin specifically inhibits initiation of RNA chains, and the initiation site may also be located on the β subunit, it was of interest to determine the structural and functional relationship between the initiation site and the rifampicin

binding sites. To this end, energy-transfer measurements were carried out to estimate the distance between these two sites. The results indicate that these two sites are at least 37 Å apart. Therefore, although both the initiation site and the rifampicin binding site are on the β subunit, they are not adjacent to each other. This suggests that the effect of rifampicin on RNA chain initiation is indirectly mediated through the enzyme molecule.

References

- Anthony, D. D., Wu, C.-W., and Goldthwait, D. A. (1969), *Biochemistry* 8, 246.
- Förster, T. (1947), *Ann. Phys.* 2, 55.
- Hartmann, G., Honikel, K., Knüsel, F., and Nüesch, J. (1967), *Biochim. Biophys. Acta* 145, 843.
- Heil, A., and Zillig, W. (1970), *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 11, 165.
- Hudson, E. N., and Weber, G. (1973), *Biochemistry* 12, 4154.
- Krakow, J. S., and Fronk, E. (1969), *J. Biol. Chem.* 244, 5988.
- Nixon, J., Spoor, T. C., Evans, J., and Kimball, A. P. (1972), *Biochemistry* 11, 4570.
- Parker, C. A., and Rees, W. T. (1960), *Analyst* 85, 587.
- Rabussay, D., and Zillig, W. (1969), *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 5, 104.
- Spoor, T. C., Persico, F., Evans, J., and Kimball, A. P. (1970), *Nature (London)* 227, 57.
- Stryer, L. (1965), *J. Mol. Biol.* 13, 482.
- Weber, K., and Osborn, M. (1969), *J. Biol. Chem.* 244, 4406.
- Wu, C.-W., and Goldthwait, D. A. (1969), *Biochemistry* 8, 4450, 4458.
- Wu, C.-W., and Wu, F. Y.-H. (1973a), *Fed. Proc., Fed. Amer. Soc. Exp. Biol.* 32, 645.
- Wu, C.-W., and Wu, F. Y.-H. (1973b), *Biochemistry* 12, 4349.
- Wu, F. Y.-H., and Wu, C.-W. (1973c), *Biochemistry* 12, 4343.
- Yarbrough, L., and Wu, C.-W. (1974), *J. Biol. Chem.* (in press).
- Zillig, W., Zechel, K., Rabussay, D., Schachner, M., Sethi, V. S., Palm, P., Heil, A., and Seifert, W. (1970), *Cold Spring Harbor Symp. Quant. Biol.* 35, 47.

FLUORESCENT CONJUGATES OF NATURAL AND BIOSYNTHETIC POLYNUCLEOTIDES

R. L. MCKENZIE*, D. B. MILLAR AND R. F. STEINER*

*Naval Medical Research Institute, National Naval Medical Center, Bethesda, Md. and *University of Maryland, Baltimore County, Baltimore, Md. (U.S.A.)*

(Received January 27th, 1972)

(Revised manuscript received April 25th, 1972)

SUMMARY

The fluorescence properties of acriflavine and methylbenz(a)anthracene conjugates of DNA and several biosynthetic polynucleotides have been examined and compared. Both types of label respond in a qualitatively similar way to helix \rightarrow coil transitions involving the polynucleotides. While the terminal-labeled acriflavine conjugates showed only a single fluorescence decay time, the methylbenz(a)anthracene conjugates gave decay curves which could best be fitted on the assumption of two fluorescent species, with different decay times. When labeled poly(rA) was incorporated into the rA : rU bihelical complex the extent to which rotational mobility, as measured by polarization, was lost at the early stages of complex formation differed for the two labels.

INTRODUCTION

Although the reversible binding of fluorescent dyes by nucleic acids has been the subject of intensive study for many years¹, the preparation and properties of covalent fluorescent conjugates of nucleic acids has received only limited attention. The types of conjugate which have been reported thus far are of two kinds.

The first type of conjugate, which is limited to RNAs, involves an initial oxidative rupture of the ribose ring to form presumably a dialdehyde, followed by a reaction of the Schiff's base type of an aldehyde group with acriflavine or a related dye^{2,3,20}. Since only the chain terminus is susceptible to oxidation in this way, the fluorescent label has in this case a definite and known location and its microenvironment will be that of the terminal nucleotide³. Conjugates of this class have been utilized for fluorescence polarization studies upon soluble RNA and synthetic polynucleotides^{3,20}.

In conjugates of the second type substitution occurs directly upon the bases. The reagent 7-bromomethylbenz(a)anthracene (MBA) has been introduced by Po-

Abbreviation: MBA, methylbenz(a)anthracene.

* Present address: National Bureau of Standards, Gaithersburg, Maryland.

From the Bureau of Medicine and Surgery, Research Subtask, MR011.0110.24 and MR041.06.01.0004 BOEX. The opinions and statements contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

chon *et al.*⁴ as a means of attaching a fluorescent label to DNA. Substitution occurs on adenine and guanine bases⁴⁻⁶. At low degrees of substitution the physical properties of the DNA are unaltered. It has been proposed that the mode of attachment is via a $-\text{CH}_2$ group and that, while the benz(a)anthracene group is not intercalated, it does not lie wholly exterior to the double helix, but is located within the large helical groove⁴⁻⁶.

Dipple *et al.*⁷ have recently presented evidence that, when the reaction is carried out in dimethylacetamide, the primary sites of attachment are the N-7 positions of guanine derivatives, the N-1 of adenine derivatives, and the N-3 of cytosine derivatives. However, in aqueous media, reaction was reported to occur mainly on the amino groups of these bases⁷.

The availability of two different labels, with altogether distinct sites of attachment, offers definite advantages in interpreting the dependence of the properties of the fluorescent probe upon the structural characteristics of the polynucleotide and in developing the use of fluorescent probes to detect structural transitions.

MATERIALS

The polynucleotides used in this study were purchased from Miles Laboratories, Elkhart, Ind. Acriflavine and 1,2-benzanthracene were obtained from Aldrich. Sucrose was Bureau of Standards calorimetric grade. Analytical grade reagents and glass-redistilled water were used for the preparation of all solutions. Paraformaldehyde was obtained from Fisher.

METHODS

Preparation of acriflavine conjugates

Acriflavine conjugates of poly(rA) and poly(rU) were prepared as described by Millar and Steiner³. The samples were stored at -20°C in the frozen state. Because the acriflavine-polynucleotide conjugate is somewhat labile, even at -20°C , samples were routinely reprecipitated with ethanol to remove any free dye prior to use. By application of the method described elsewhere³, it was found that between 0.5 and 1.0 groups were introduced per molecule of poly(rA) or poly(rU) for the conjugates cited here.

Preparation of MBA-poly(A) and MBA-DNA conjugates

7-Bromomethylbenz(a)anthracene was prepared by the method of Badger and Cook⁸. Poly(rA) conjugates were prepared by the method of Pochon and co-workers^{4,5}. After completion of the reaction the conjugate was precipitated with ethanol from 0.5 M KCl. The degree of labeling was determined by assuming molar extinction coefficients for poly(rA) and MBA of 10^4 and $5 \cdot 10^4$ at 260 and 360 nm, respectively. MBA-poly(rA) I was labeled to the extent of one residue in 800 and MBA-poly(rA) II to the extent of one residue in 900.

Concentrations of polynucleotides were calculated from experimentally determined molar absorption coefficients for the buffer system being used. Molar absorption coefficients for each polynucleotide conjugate were determined by meas-

using the concentration of either snake venom and/or 0.1 M KOH hydrolysis (≥ 18 h at 37°C) by measuring $A_{260\text{ nm}}$. The absorbance at 260 nm of the polynucleotide conjugate could then be measured and the corresponding $\epsilon_{260\text{ nm}}$ for that conjugate determined. All polynucleotide conjugates had ϵ values in the range $9.6 \cdot 10^4$ to $11 \cdot 10^4$.

The MBA conjugate of calf thymus DNA was prepared by a similar procedure except that the lyophilization step was omitted. The three DNA conjugates cited here, MBA-DNA IV, V, and VI, were labeled to the extent of one residue in 1700, 1300 and 220, respectively.

Fluorescence polarization

The anisotropy r of emitted light¹⁰ is defined by:

$$r = \frac{3}{2} \frac{(I_{\parallel} - I_{\perp})}{(I_{\parallel} + 2I_{\perp})}$$

where I_{\parallel} and I_{\perp} are the respective intensities of the vertically and horizontally polarized components of the emission when the exciting light is vertically polarized. This procedure leads to a simplification of expressions relating to depolarization.¹⁰ The familiar Perrin equation, which refers to a sphere of volume V undergoing Brownian rotation in a medium of viscosity η at a temperature T (°K), now assumes the form:

$$r^{-1} = r_0^{-1} \left(1 + \frac{K\tau}{\eta V} \right) \quad (1)$$

where τ is the lifetime of the excited state; K is the Boltzmann constant; and r_0 is the anisotropy in the absence of rotation. Substituting in the above equation the expression $r_0 = 3\eta V / KT$, where r_0 is the rotational relaxation time, gives

$$r^{-1} = r_0^{-1} \left(1 + \frac{3\tau}{\rho_0} \right) \quad (2)$$

Determination of μ_0 through a Perrin plot, as introduced by Weber¹¹ allows the determination of ρ_0 , subject to the often doubtful assumption that V , μ_0 , and the molecular shape are independent of temperature and viscosity.

Static emission anisotropy measurements in our laboratory were made using a Phoenix light scattering photometer, modified as described by Millar and Steiner.¹² A further modification was made by the use of a mercury-xenon arc (Hanovia 901-C). Light from the lamp was passed successively through a prism monochromator and a Polacoat polarizer, so that the light incident on the sample was vertically polarized. A second polarizer, placed before the photomultiplier, intercepted the fluorescent beam.

Fluorescence intensity

The intensity of emission was obtained from emission anisotropy data and is equal to $I_{\parallel} + 2I_{\perp}$. Emission and excitation spectra were obtained upon either an Aminco or a Turner 210 spectrofluorimeter.

* As discussed by Jablonski¹⁰ the anisotropy is a better measure of the angular dissymmetry of the emitted light than is polarization. It may be noted that the definition of anisotropy employed by some authors is simply related to ours by a factor of two-thirds.

Fluorescence lifetimes

Some of the excited lifetime measurements reported here were made with a TRW model 75A decay time fluorimeter with a 31B nanosecond spectral source and a type 556 Tektronix dual-beam oscilloscope. Measurements were also made using the nanosecond time decay fluorimeters of Dr Irvin Isenberg of Oregon State University and Dr Ludwig Brand of Johns Hopkins University, to whom we are also indebted for their help and interest. The assistance of Dr Robert Schuyler is also gratefully acknowledged.

RESULTS

Both the above instruments utilize single photon counting¹². In both cases, analysis of data was made by the method of moments^{12,14}. The parameters yielding the best fits on the assumption of one or two fluorescent components were used to generate computed decay curves, which were compared directly with the experimental curves.

Polyriboadenylic acid

The variation with pH of fluorescence intensity, anisotropy, and ultraviolet absorbance of a MBA conjugate of poly(rA) is shown in Fig. 1. Both fluorescence intensity and anisotropy indicate a transition at pH 5.8 corresponding to the transition from the alkaline form to the bihelical acid form of poly(rA). The transition, as measured by these parameters, shows significantly greater breadth than that indicated by absorbance measurements and suggests the possible existence of inter-

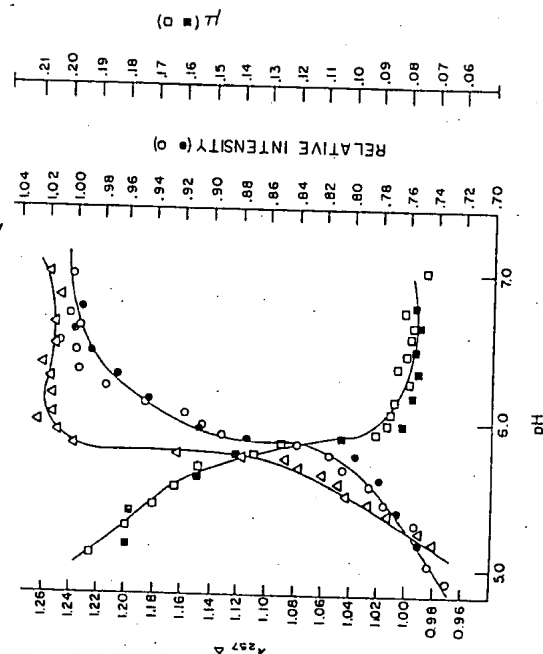


Fig. 1. The acid pH dependence of relative fluorescence intensity (O), absorbance at 257 nm (Δ) and emission anisotropy (□) for MBA-poly(rA) II in 0.5 M KCl, 0.001 M phosphate at 20°C. The excitation wavelength is 366 nm. Filled symbols represent reversals.

mediate states, as was observed by Millar and Steiner for acriflavine-poly(rA) conjugates³.

The increase in emission anisotropy, indicating an increased rotational relaxation time at acid pH is expected for the more ordered bihelical structure. The relative fluorescence intensity decreases with the transition to the acid form. However, as Fig. 2 shows, no significant spectral shifts occur upon going from pH 7 to 5.

Table I cites values for the apparent excited lifetimes of MBA and acriflavine

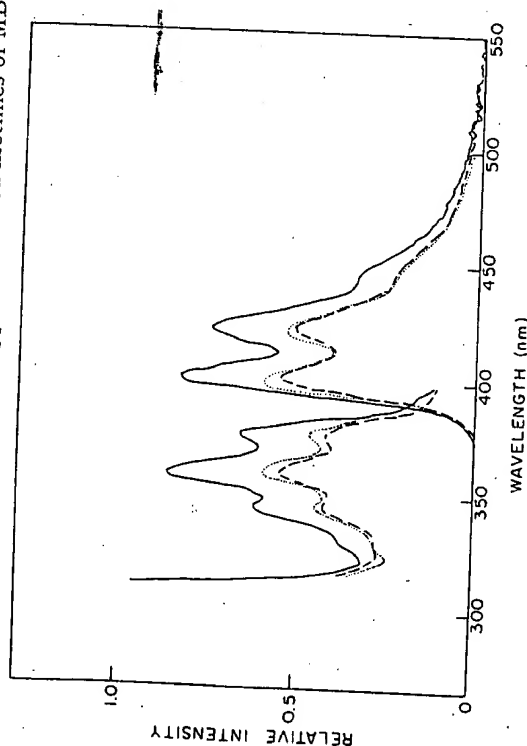


Fig. 2. The excitation and emission spectra of MBA-poly(rA) II at pH 7.0 (—) and at pH 5.0 (---) and those of the rA : rU complex of MBA-poly(rA) II (···). The solvent is 0.5 M KCl, 0.001 M phosphate at 25°C. The poly(rA) concentration is 1.5 mg/ml. The excitation and emission wavelengths are 360 and 400 nm, respectively.

TABLE I

VALUES OF EXCITED LIFETIMES AND APPARENT RELAXATION TIMES FOR POLY(rA) AND POLY(rU) CONJUGATES

The solvent is 0.5 M KCl, plus 0.01 M potassium phosphate (pH 7) or 0.01 M potassium acetate (pH 5).

Conjugate	pH	τ (ns)		Instrument	ρ^* (ns)
		1-component	2-component		
MBA-poly(rA) 1	7.0	28.5		Isenberg	
MBA-poly(rA) 2	7.0	Approx 25**		TWR	27
	7.0	28.2	29.4 (80 %)	Brand	
			11.1 (20 %)		
Acriflavine-poly(rA)	5.0	27.8	32.2 (62 %)	Brand	147
			14.8 (38 %)		
	7.0	5.6		TWR	16
Acriflavine-poly(rU)	5.35	5.6	5.7 (99 %)	Isenberg	
	7.0		5.6 (>99 %)	TWR	36
				Isenberg	

* From Perrin plot, assuming τ constant and equal to average (1-component) value

** Best visual fit.

... Percentage contribution of component of indicated lifetime.

conjugates of poly(rA), as measured on several different instruments. It was found that, at both pH 7.0 and 5.0, the experimental curves of decay of intensity with time could be significantly better fitted on the basis of two fluorescence decay times than on the basis of a single decay time (Table I and Fig. 3). Decay times computed according to both models are cited in Table I. There is some indication of an increase at pH 5.0 of the contribution of the component of shorter lifetime (Table I).

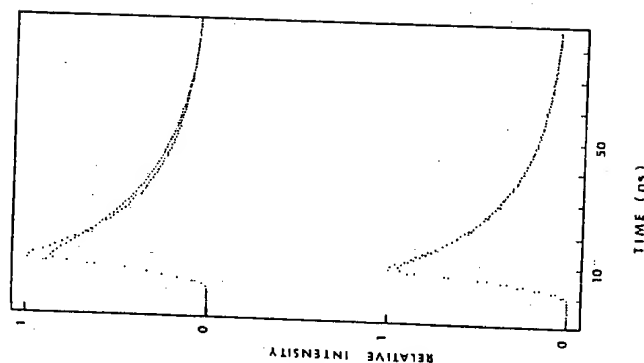


Fig. 3. Time decay of fluorescence intensity for MBA-poly(rA) II when incorporated into a 1 : 1 rA : rU complex in 0.5 M KCl, 0.01 M phosphate (pH 7.0) at 25°C. Lower: a comparison of data with best 2-component fit. Upper: best 1-component fit. Data were obtained with the nanosecond fluorimeter of Dr L. Brand.

In contrast, acriflavine-poly(rA) showed virtually exponential decay at both pH's with no indication of a change in lifetime with pH (Table I).

Fig. 4 shows Perrin plots of MBA-poly(rA) at pH 7.0 and 5.0. The values of $T\eta$ were altered by the addition of a sucrose stock solution of the same pH and ionic strength as the original buffer of the sample, while maintaining the temperature at 20°C. Apparent rotational relaxation times were determined using Eqn 3 and the results are cited in Table I. The increased rotational relaxation time at pH 5 is in harmony with the expected greater structural rigidity of the acid bihelical form in comparison with the alkaline form¹⁵⁻¹⁸.

The decrease in intensity of fluorescence of MBA-poly(rA) noted in Fig. 2 and the absence of spectral shifts suggest that a decrease in the lifetime of the excited state may be occurring. The lifetime measurements cited indicate that such is the case.

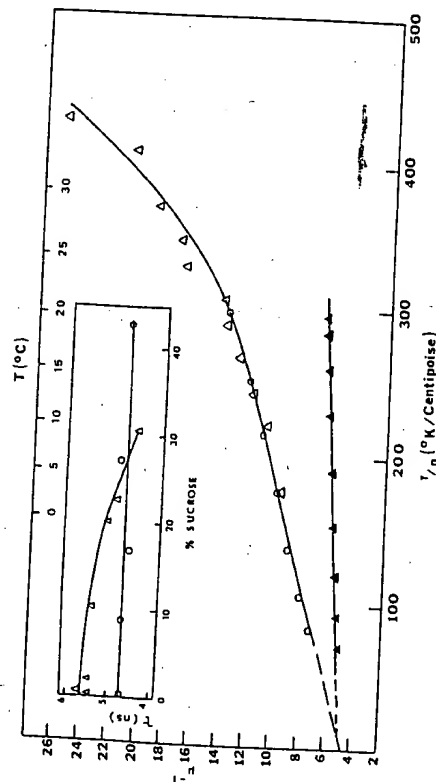


Fig. 4. Combined Perrin plots for MBA-poly(rA) I at pH 7.0, 20°C, and varying sucrose level (○); pH 7.0 in the absence of sucrose and varying temperature (Δ); and at pH 5.0, 20°C, and MBA-poly(rA) I is 1 mg/ml. The solvent is 0.5 M KCl, 0.001 M phosphate. The concentration of the excited lifetime of acriflavine-poly(rA) is 366 nm. Inset: The dependence of T/η level in 0.5 M KCl, 0.001 M phosphate (pH 7.0).

A 10 % decrease in τ was observed on going from 0 to 30 % sucrose. A calculation of the expected magnitude of the effect of this change on the value of relaxation time, using the method of Johnson and Thornton¹⁹, indicates that, for the present case, the correction amounts to less than 5 %.

In the temperature range of these experiments the fluorescent label does not appear to develop any new degrees of rotational freedom. Fig. 4 shows Perrin plots for MBA-poly(rA) in which T/η was varied both by adding sucrose at a constant temperature and by varying the temperature for constant solvent conditions. In the region of overlap the values are identical, suggesting that μ is a function of T/η . The slight upward curvature observed above 25°C may be interpreted as the appearance of new, thermally induced degrees of rotational freedom in this temperature range. To test for the possibility that detachment of the label might occur at higher temperatures, a sample was maintained at 25°C for 48 h. No change in μ occurred in this period and subsequent heating to 40°C for 30 min likewise produced no change in μ . This stability of the MBA conjugates, which contrasts with the acriflavine conjugates, renders them particularly useful for monitoring temperature effects.

As pointed out earlier, acriflavine conjugates can be prepared only with the purine base along the length of the chain, whereas the MBA label may be present on any single (> 99 %) fluorescent species having a lifetime of 5.67 ns. This homogeneity is excepted in view of the specificity of label position.

Measurements with the TWR apparatus gave a value of τ of 5.62 ± 0.07 ns for acriflavine-poly(rA) (Table I), where the value is an average of 12 determinations.

and the error corresponds to 2 standard deviations from the average. This value is very close to that found for acriflavine-poly(rU) and suggests that the nature of the base does not influence the acriflavine lifetime.

As with MBA-poly(rA), the lifetime was influenced by the sucrose concentration of the solution. Fig. 4 shows the effect on τ of added sucrose. In contrast, the free dye shows no quenching by sucrose. This suggests that quenching may occur from indirect effects on the polymer itself, rather than by direct interaction with acriflavine.

The transition profile from the alkaline to the acid form of acriflavine-poly(rA), as measured by emission anisotropy by Millar and Steiner³ is in close agreement with the transition profile reported here for MBA-poly(rA). There are thus no obvious differences in behavior between the terminus and the bulk of the polymer. Determinations of the rotational relaxation times of the acid and alkaline forms of acriflavine-poly(rA) are likewise in agreement with expectations of greater structural rigidity for the acid bihelical form (Table I). The apparent relaxation times of both forms of poly(rA) are lower for the acriflavine than for the MBA conjugate.

Poly(rU)

The effect of temperature upon the emission anisotropy of acriflavine-poly(rU) is shown in the form of a Perrin plot in Fig. 5. The appearance of upward curvature at about 5°C, indicating new rotational freedom, correlates well with the absorbance change at 259 nm, reported by Lipsett²⁰, which was attributed to the thermal disruption of the organized helical form prevailing at low temperatures, the melting point being close to 5-8°C.

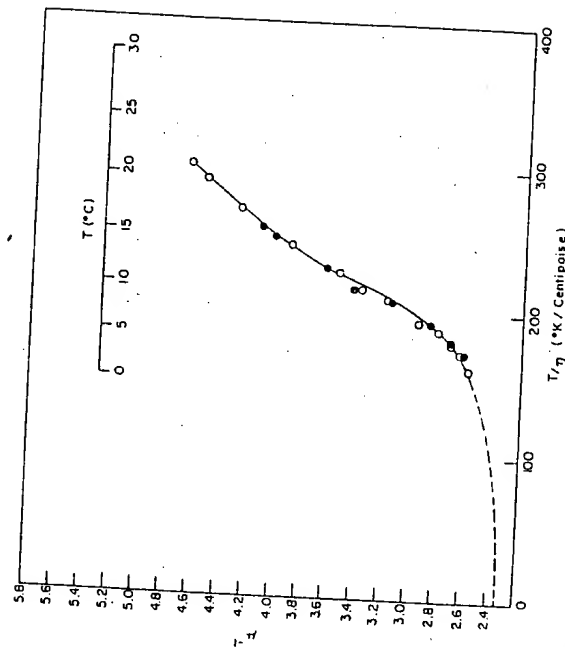


Fig. 5. The temperature dependence of the emission anisotropy of acriflavine-poly(rU) (1 mg/ml) in 0.5 M KCl, 0.001 M phosphate (pH 7.0). Filled symbols represent reversals.

Interaction of poly(rA) and poly(rU)

The effect of the addition of poly(rU) to a solution of acriflavine-poly(rA) on the absorbance at 260 and 280 nm and on the intensities and emission anisotropies of fluorescence are shown in Figs 6 and 7. The changes in absorbance reflect the formation of a bihelical rA:rU complex at a poly(rU) mole fraction of 0.5 and a trihelical rA:2rU species at a mole fraction of poly(rU) of 0.67 as reported by Stevens and Felsenfeld²⁴. For both acriflavine-poly(rA) and MBA-poly(rA) conjugates, a biphasic behavior of anisotropy is noted in the mixing curves, with a definite inflection point occurring at a poly(rU) mole fraction of 0.5, followed by a further increase in emission anisotropy up to a mole fraction of 0.67.

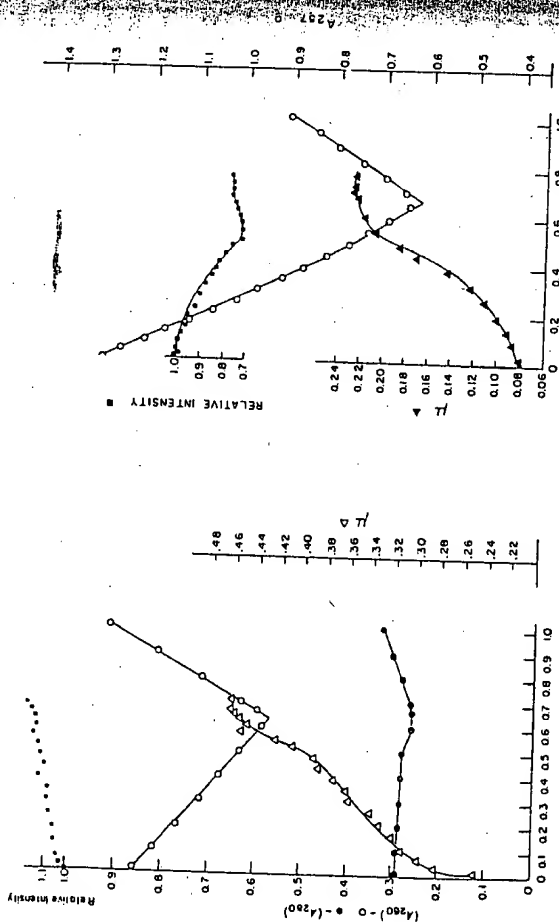


Fig. 6. The variation with mole fraction of poly(rU) of fluorescence intensity (●), emission anisotropy (Δ), absorbance at 280 nm (●) and at 260 nm (○) for acriflavine-poly(rA) (10^{-4} M) in 0.5 M KCl, 0.001 M phosphate (pH 7.0) at 25°C.

Fig. 7. The variation with mole fraction poly(rU) of emission anisotropy (▲), fluorescence intensity (■), and absorbance at 257 nm (○) for MBA-poly(rA) in 0.5 M KCl, 0.001 M phosphate at 25°C. The nucleotide concentrations were 10^{-4} M and 0.7 mg/ml for absorbance and fluorescence measurements, respectively.

The corresponding profile of relative fluorescence intensity versus mole fraction of poly(rU) is different for the two types of conjugate. For MBA-poly(rA) a definite inflection point is observed at a mole fraction of poly(rU) of 0.5. The observed variation is consistent with the consecutive formation of the rA:rU and rA:2rU species. In contrast, the acriflavine-poly(rA) conjugate shows a gradual increasing intensity with increasing mole fraction of poly(rU), but does not display any obvious inflection at a mole fraction of 0.5. These differences may arise from either the nature of the fluorescent label itself or from the effects of differing positions of the label.

A comparison of the profiles of emission anisotropy as a function of mole frac-

tion of poly(rU) indicates that a significant difference between the two conjugates occurs in the initial portion of the curve, up to a poly(rU) mole fraction of about 0.3. For acriflavine-poly(rA) the emission anisotropy increases more gradually up to a mole fraction of 0.5. In contrast, the emission anisotropy of MBA-poly(rA) increases more gradually up to a mole fraction of poly(rU) of about 0.3 and then more sharply to a mole fraction of 0.5. Both types of conjugate then show a moderately rapid increase beginning at a mole fraction of 0.5, followed by a levelling off and saturation at a mole fraction of 0.67.

Formation of the rA:rU and rA:2rU species results in a significant decrease in mean lifetime of the MBA conjugate with a corresponding increase in the contribution of the short lifetime component (Table II). In contrast, the lifetime of the acriflavine conjugate was essentially unchanged, within experimental uncertainty, over the entire range of compositions.

TABLE II
VALUES OF EXCITED LIFETIMES AND APPARENT RELAXATION TIMES FOR rA:rU AND rA:2rU CONJUGATES

Conjugate	Species	τ (ns)		Instrument	ρ^* (ns)
		1-component	2-component		
MBA-poly(rA) 2	rA:rU	24.7	32.1 (39.%)**	Brand	206
	rA:2rU	21.3	15.7 (61.%)	Brand	Approx. 3500
Acriflavine-poly(rA)	rA:rU		26.7 (44.%)	Brand	
			10.0 (56.%)		> 92

* Computed from Perrin plots, using average (1-component) value of τ . The solvent is the same as for Table I.

** Percentage contribution of component of indicated lifetime.

The increase in emission anisotropy for the rA:rU and rA:2rU species indicates an increase in apparent relaxation time. The values of relaxation time are too long in comparison with the excited lifetime to permit computation of more than approximate values, as the slopes of the Perrin plots are close to zero. A major increase in relaxation time accompanies the transition from the rA:rU to the rA:2rU species (Table II).

Analysis of poly(rA)-poly(rU) mixing curves

The method developed by Evett *et al.*⁹ and by Ellerton and Isenberg²² permits calculation of the fraction of labeled polymer which has acquired the characteristics of the complex species as a function of the mole fraction of poly(rU).

It is assumed that the fluorescent conjugate exists in either of two states, corresponding to those existing before and after the transition of interest. Subscripts 1 and 2 will designate the initial and final parameters, respectively.

Let Φ_1 , Φ_2 = fractions of light emitted by Species 1 and 2, respectively and f = fraction of fluorescent moieties having undergone the transition from State 1 to State 2.

If it is assumed that the solution is dilute enough so that the fluorescence intensity (I) is proportional to ϵqN , where ϵ is the molar extinction coefficient, q is the

quantum yield, and N is the number of fluorescent molecules, and that ϵ and q for the fluorescent species which have not undergone the transition are unaffected by the presence of poly(rU), a derivation similar to that of Ellerton and Isenberg²¹ gives the relations:

$$f_0 \rightarrow 1:1 = 1 + \frac{I_{\text{ra}}(\mu_2 - \mu_1)}{I_0(\mu_2 - \mu_1)}; 0 < X_u < 0.5$$

and

$$f_1 \rightarrow 1:1:2 = 1 + \frac{I_{\text{ra}}(\mu_2 - \mu_1)}{I_{0,2}(\mu_2 - \mu_1)}; 0.5 < X_u < 0.67$$

where I_{ra} = intensity corresponding to a particular value of X_u . In the former equation State 1 corresponds to $X_u = 0$; and State 2 to $X_u = 0.5$; in the latter State 1 corresponds to $X_u = 0.5$ and State 2 to $X_u = 0.67$.

The justification for dividing the mixing curve into two independent regions lies in the demonstration that only the rA:rU species is formed up to values of X_u of 0.5 and that only at higher values of X_u is the rA:2rU species formed^{23,25,26}. Fig. 8 shows $f_0 \rightarrow 1:1$ and $f_1 \rightarrow 1:1:2$ as a function of X_u . The dashed line indicates the fraction of the bases of poly(rA) which have undergone the transition and is based on the known stoichiometry of the interaction and upon the variation in $f_0 \rightarrow 1:1$ and $f_1 \rightarrow 1:1:2$ which is computed from the change with X_u of the absorbance at 257 or

It is clear that, while the variation in $f_1 \rightarrow 1:1:2$ is consistent with the predicted curve, significant deviations occur in the case of $f_0 \rightarrow 1:1$ for $0 < X_u < 0.5$. Moreover, there are some differences in behavior between the acriflavine-poly(rA) and MBA-poly(rA) conjugates, which show opposite deviations from the predicted curve.

This kind of behavior is not unexpected, inasmuch as the fluorescent labels are not measuring the state of the entire polymer, but rather of segments. The results shown in Fig. 8 are consistent with, and suggest, that the terminal regions of the poly(rA) molecule, which are monitored by the acriflavine label, lose rotational

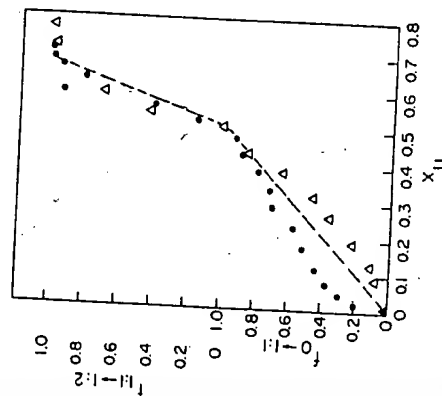


Fig. 8. The fraction of conjugate which has undergone the transition from poly(rA) to rA:rU, $f_0 \rightarrow 1:1$, and from rA:rU, $f_1 \rightarrow 1:1:2$, for acriflavine-poly(rA) (●) and for MBA-poly(rA) (Δ). The dashed line indicates the fractional transition based on the stoichiometry of interaction.

Biochim. Biophys. Acta, 277 (1972) 306-322

freedom to a greater degree at low extents of formation of the rA:rU species than does the bulk of the polymer, whose behavior is reflected by the MBA label. Once the rA:rU species is formed the differences between the terminus and the balance of the molecule disappear and their behavior becomes coincident.

DNA-MBA conjugates

Dependence of quantum yield upon pH for a DNA-MBA conjugate in the presence of 0.1 M KCl is illustrated in Fig. 9 and 10. In the acid pH range a significant quenching begins below pH 6 and increases down to about pH 3. At still lower pH's a major enhancement of fluorescence with decreasing pH occurs (Fig. 9).

In the alkaline pH range, under the same conditions a significant quenching begins at about pH 11, becoming maximal at about pH 11.7. At higher pH's a rapid

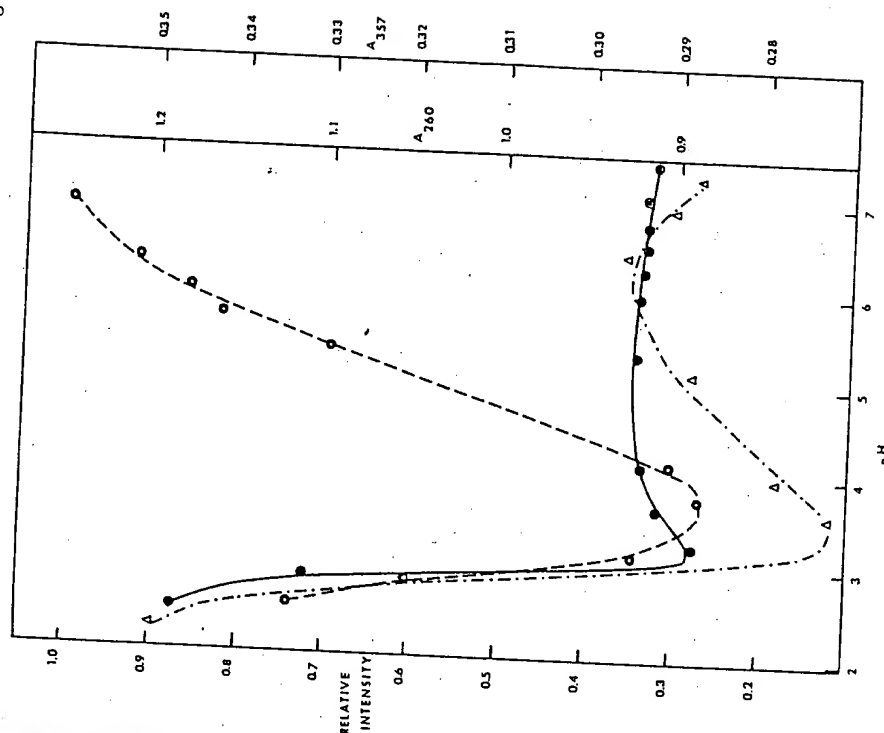


Fig. 9. Acid pH dependence of relative fluorescence yields (O), absorbance at 357 nm (Δ) for MBA-DNA VI in 0.1 M KCl, 0.001 M phosphate at 25°C. The concentrations for fluorescence and for absorbance at 357 nm are 0.5 and 2 mg/ml, respectively. The excitation wavelength is 340 nm.

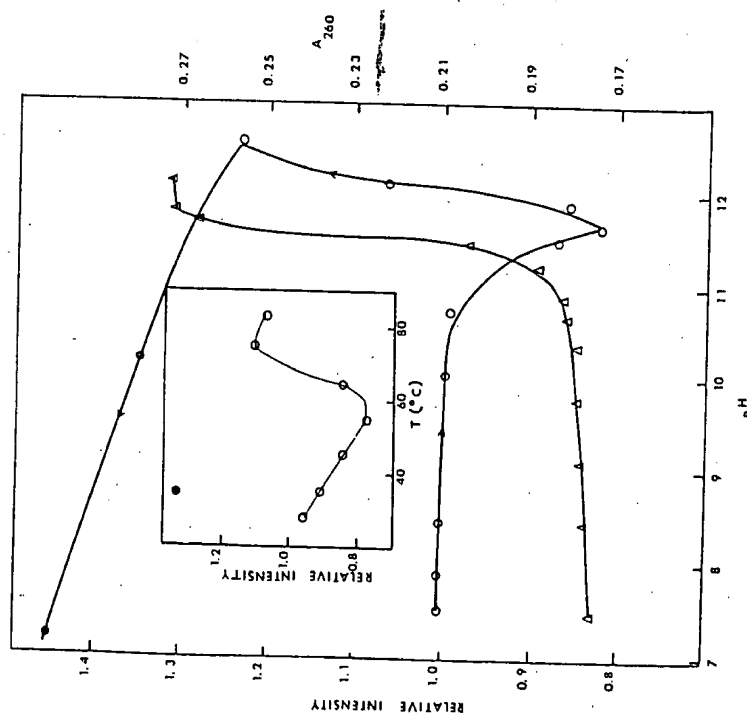


Fig. 10. The alkaline pH dependence of relative fluorescence yield (O) and of absorbance at 260 nm (Δ) for MBA-DNA VI in 0.1 M KCl, 0.001 M phosphate at 25°C. The concentration of MBA-DNA VI for fluorescence is 0.6 mg/ml. The excitation wavelength is 340 nm. The filled circles represent data obtained by back titration from alkaline pH. Inset: Temperature dependence of emission intensity for MBA-DNA IV (0.5 mg/ml) in 0.001 M phosphate (pH 7.5). The excitation wavelength is 340 nm. The filled point represents a reversal.

increase in fluorescence with increasing pH occurs, in parallel with the behavior at pH's below 3. This is accompanied by a shift to shorter wavelengths of the peak positions, the positions of the two maxima shifting from 399 and 413 nm to 396 and 411 nm (Fig. 11). Back titration from pH 12.5 does not reproduce the original curve, but results in relative quantum yields at neutral pH which are considerably in excess of the initial values (Fig. 10).

The fluorescence changes are accompanied by changes in the absorption spectrum of the MBA group. A pronounced positive difference spectrum is developed at pH's above 12 with respect to neutral pH (Fig. 11). A positive difference spectrum is likewise developed at acid pH, the pH profile being correlated with the increase in fluorescence intensity below pH 3 (Fig. 9).

The disruption of the native bihelical form of DNA by exposure to extremes of pH is accompanied by a loss of hypochromism, as reflected by an increase in absorbance at 260 nm. In the acid pH range this is closely correlated with the increase in fluorescence intensity occurring below pH 3, while in the alkaline range its midpoint occurs in the zone of quenching (Figs 9 and 10).

Biochim. Biophys. Acta, 277 (1972) 306-322

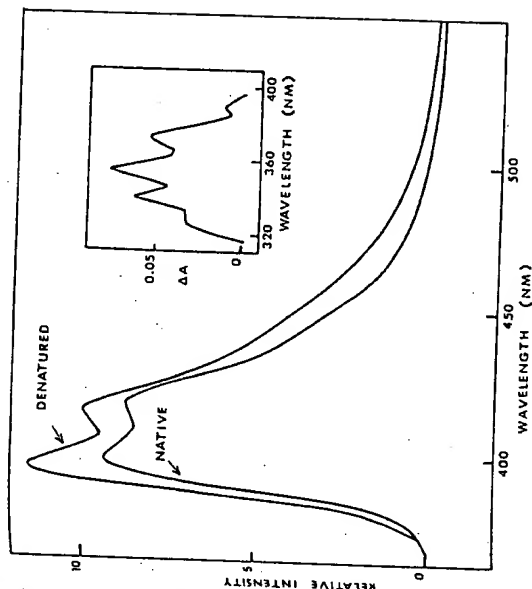


Fig. 11. Emission spectra of native (pH 7.0) and alkaline denatured (pH 12.5) forms of MBA-DNA VI (0.5 mg/ml) in 0.1 M KCl, 0.001 M phosphate. The excitation wavelength is 340 nm. Inset: Difference spectrum (pH 12.9 vs pH 7.5) of the MBA label of MBA-DNA VI (4 mg/ml) in 0.1 M KCl, 0.001 M phosphate.

The acid and alkaline pH profiles of relative fluorescence intensity thus both display an initial quenching, followed by a rise in quantum yield at extremes of pH. In the case of the acid branch the decrease in intensity becomes important at much too high a pH to be identified with the classical acid denaturation of DNA, which results in a loss of hypochromism. It must therefore, result, at least in part, from either a structural transition below pH 6, or else from a direct quenching effect of the protonated adenine and cytosine groups. The rise in quantum yield below pH 3 clearly corresponds to the acid-induced helix \rightarrow coil transition.

The alkaline branch is likewise biphasic. Here too, it is difficult to separate the effects of base ionization from those of loss of bihelical structure. However, the enhancement in intensity occurs in the same pH range in which actual strand separation, as reflected by a drop in molecular weight, occurs at this ionic strength, as reported by Studier²⁷.

The temperature profile of the relative quantum yield in 0.001 M potassium phosphate buffer (pH 7.5) is shown in Fig. 10. An initial monotonic decrease is followed above 65°C by an abrupt increase. Upon rapid cooling to room temperature, the enhancement in fluorescence efficiency persists. The initial decrease in intensity with increasing temperature presumably represents normal thermal quenching, while the subsequent enhancement arises from the transition to the randomly coiled, denatured form of DNA.

Excited Lifetime

Measurements with both the TRW and the instrument of Isenberg indicated

Biochim. Biophys. Acta, 277 (1972) 306-322

a complex and non-exponential decay curve, making it impossible to cite reliable values of the lifetimes.

Effect of external perturbants upon the quantum yield

The significant responses of MBA-DNA to the quencher, KI, and to the non-quenching perturbant, ethylene glycol, neither of which is enhanced by disruption of the bihelical structure by thermal denaturation (Fig. 12), makes it likely that the MBA residues are not intercalated within the stacked helical bases of the native form of DNA and that their accessibility to perturbant is not significantly blocked through their involvement in the DNA double helix.

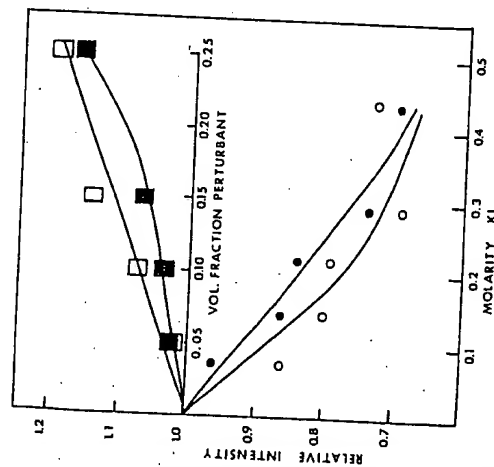


Fig. 12. Upper: Dependence of relative fluorescence yield upon volume fraction of ethylene glycol for the native (\square) and denatured (\circ) forms of MBA-DNA VI (2 mg/ml) in 0.1 M KCl, 0.001 M phosphate (pH 7.5) at 25°C. Lower: Dependence of relative fluorescence yield upon molarity of KI for the native (\circ) and denatured (\bullet) forms of MBA-DNA V (0.08 mg/ml) in 0.1 M KCl, 0.001 M phosphate (pH 7.5) at 25°C. For both upper and lower curves, the excitation wavelength was 340 nm. Thermally denatured DNA was produced by heating at 100°C for 15 min.

DISCUSSION

The quantum yield and anisotropy of both types of conjugate are sensitive to structural transitions of the polynucleotide chain and provide a means of monitoring such transitions.

In contrast to the acriflavine conjugates, MBA conjugates of poly(rA) show more than one fluorescence decay time, indicating a heterogeneity of microenvironment of the label or differing positions of attachment to the base. In this case a significant difference in lifetime is noted for the acid and alkaline forms.

Although Dipple *et al.*⁷ have reported that the amino groups are the primary sites of attachment when the reaction is carried out in an aqueous medium, it is

Biochim. Biophys. Acta, 277 (1972) 306-322

difficult to exclude the possibility of some degree of attack at the N-7 position. A mixture of both species could explain the presence of two decay times. Alternatively, the relation of the fluorochrome to the regions of stacked bases may vary, since more than one orientation is possible.

Perrin plots for both acriflavine and MBA conjugates of the alkaline form of poly(rA) yield similar pictures of the conformational state of the polymer. Considerable caution is of course necessary in interpreting Perrin plots for systems of this kind, where several of the assumptions involved in derivation of the Perrin equation, including especially those of random orientation of the label and absence of free rotation of the label, may not be valid. The relaxation times obtained in this way should thus be regarded as only apparent values. Nevertheless, it appears clear that the values of relaxation time obtained for either conjugate are much too small to be consistent with a rigid molecule having the dimensions of the polynucleotide chain. The equivalence of anisotropy values obtained as a function of T/η by adding sucrose label is not a major factor in this temperature range. The comparable values of relaxation time obtained for MBA and acriflavine conjugates of alkaline poly(rA) indicate that the terminal nucleotide is not atypical with respect to rotational freedom.

The apparent values of relaxation time for alkaline poly(rA) are, however, large in comparison with that expected for a single nucleotide and indicate a significant degree of rigidity of the polynucleotide strand. Indeed the observed values would be formally equivalent to a rotational kinetic unit containing 100 nucleotides.

Results with both labels thus agree that the single-stranded, interrupted helical form characteristic of alkaline poly(rA) possesses significant structural rigidity, although the average rotational kinetic unit does not encompass a major fraction of the molecule.

The major increase in apparent relaxation time observed with conjugates of both types upon making the transition to the acid form of poly(rA) is consistent with the increase in rigidity expected in view of the double stranded helical nature of the latter. Here a significant difference is observed for the two conjugates, those of the acriflavine type having a somewhat lower relaxation time, perhaps as a consequence of increased flexibility of the terminus.

The interaction of poly(rA) with poly(rU) is, as would be expected, accompanied by a major increase in apparent relaxation time, corresponding to the transition to a more rigid bi- or tri-helical structure. With both types of label a major difference is noted between the rA : rU and rA : rU helical species, the latter having a much higher apparent relaxation time. It seems clear that the bi-helical rA : rU complex does not approach complete structural rigidity, which requires the addition of a third strand. As there is no evidence for any significant interruption of the bi-helical structure, it is likely that this reflects an intrinsic flexibility of the bi-helical structure itself.

Very little rotational freedom of the MBA label appears to persist for the rA : rU species. If the preferred site of attachment is indeed the 6-amino group of adenine it would be expected that this might interfere with the formation of a stable base pair. Apparently this does not result in sufficient local flexibility to affect the rotational mobility of the label to a major degree.

The results with MBA conjugates of DNA are consistent with, and suggest a

Biochim. Biophys. Acta, 277 (1972) 306-322

relatively high degree of exposure to solvent, as is reflected by its response to per-turbants and quenchers. This would be the case if the MBA groups were located within the wide groove of the bi-helical structure, rather than intercalated within the stacked bases.

Nevertheless, the microenvironments of the MBA groups appear to exert a significant influence upon their spectral and emission properties, as is manifested by the pronounced changes accompanying the helix \rightarrow coil transition. Loss of the helical structure results in an increase in fluorescence yield, a shift in emission wavelength, and the development of a difference spectrum.

ACKNOWLEDGEMENT

R. McK. is a resident research associate, National Research Council, National Academy of Sciences, 1970-1971.

REFERENCES

- 1 L. S. Lerman, *Proc. Natl. Acad. Sci. U.S.A.*, **49** (1963) 94.
- 2 J. Churchich, *Biochim. Biophys. Acta*, **75** (1963) 274.
- 3 D. B. S. Millar and R. F. Steiner, *Biochim. Biophys. Acta*, **102** (1965) 571.
- 4 F. Pochon, P. Brookes and A. M. Michelson, *Eur. J. Biochem.*, **21** (1971) 154.
- 5 F. Pochon and A. M. Michelson, *Eur. J. Biochem.*, **21** (1971) 144.
- 6 F. Pochon, A. M. Kapular and A. M. Michelson, *Proc. Natl. Acad. Sci. U.S.A.*, **68** (1971) 205.
- 7 A. Dipple, P. Brookes, D. S. Makintosh and M. P. Rayman, *Biochemistry*, **10** (1971) 4323.
- 8 G. M. Badger and J. W. Cook, *J. Chem. Soc.*, (1939) 802.
- 9 J. Evett, R. L. McKenzie and I. Isenberg, *Biochemistry*, **9** (1970) 4573.
- 10 A. Jablonski, *Bull. Acad. Pol. Sci., Ser. Sci. Math. Astr. Phys.*, **8** (1960) 259.
- 11 G. Weber, *Biochem. J.*, **51** (1952) 145.
- 12 R. Schuyler and I. Isenberg, *Rev. Sci. Instrum.*, **42** (1971) 813.
- 13 I. Isenberg and R. D. Dyson, *Biochem. J.*, **9** (1969) 1337.
- 14 R. D. Dyson and I. Isenberg, *Biochemistry*, **10** (1971) 3233.
- 15 R. F. Steiner and R. F. Beers, *Biochim. Biophys. Acta*, **32** (1959) 166.
- 16 D. N. Holcomb and S. N. Timasheff, *Biopolymers*, **6** (1968) 513.
- 17 H. Eisenberg and F. Felsenfeld, *J. Mol. Biol.*, **30** (1967) 17.
- 18 D. N. Holcomb and I. Tinoco, *Biopolymers*, **3** (1965) 121.
- 19 P. Johnson and A. C. R. Thornton, *Biochem. J.*, **115** (1966) 725.
- 20 M. N. Lipsett, *Proc. Natl. Acad. Sci. U.S.A.*, **46** (1960) 445.
- 21 C. L. Stevens and G. Felsenfeld, *Biopolymers*, **2** (1964) 293.
- 22 N. F. Ellerton and I. Isenberg, *Biopolymers*, **8** (1969) 767.
- 23 G. Felsenfeld and A. Rich, *Biochim. Biophys. Acta*, **26** (1957) 457.
- 24 G. Felsenfeld, *Biochim. Biophys. Acta*, **29** (1958) 133.
- 25 J. Massoulié, *Eur. J. Biochem.*, **3** (1968) 428.
- 26 J. Massoulié, *Eur. J. Biochem.*, **3** (1968) 439.
- 27 F. W. Studier, *J. Mol. Biol.*, **11** (1965) 373.
- 28 D. B. S. Millar and R. F. Steiner, *Biochemistry*, **5** (1966) 2289.

Biochim. Biophys. Acta, **277** (1972) 306-322

STUDIES ON TRANSFER RNAs

II. MODIFICATION OF *ESCHERICHIA COLI* FORMYLMETHIONINE TRANSFER RNA

YOSHIYUKI KAWAMURA AND YOSHIHISA MIZUNO

Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo (Japan)
(Received March 9th, 1972)

SUMMARY

1. Treatment of tRNA^{Met} with hydroxylamine caused specific modification of cytosine residues among a number of constituent nucleobases and only three out of the 26 cytosine residues in the tRNA were modified. The modified tRNA was found to charge methionine up to 64 % of its original acceptance.

2. By base-sequence analysis of the modified tRNA, it was found that C₁ (the C residue at position 1 counting from the 5'-end), C₁₆ in the dihydrouridine loop and C₇₆ in the 3'-terminus were modified to the extent of 70, 50 and 80 %, respectively.

3. As compared with earlier findings on the chemical modification experiments of tRNA, a striking result of the present work is that a cytosine residue in the anticodon CpApUp was resistant to this modification.

INTRODUCTION

Chemical as well as enzymatic modifications of tRNA may be promising tools for studying the interrelationship of the structure and function of tRNA. The method of chemical modification should yield a tRNA with chemical alternations of known distribution, extent and character.

The reaction of hydroxylamine with cytosines has been well studied, largely because of an interest in the mutagenic action of this reagent whose action is primarily associated with the modification of cytosine residues in RNAs or DNAs, especially tRNAs¹⁻⁴.

The hydroxylation of cytosines may give rise to the corresponding N⁴-hydroxycytosine derivatives as well as 5,6-dihydro-N⁴-hydroxy-6-hydroxylamino-cytosine derivatives. This reaction is quite specific for cytosines, provided that the reaction is carried out in acidic regions^{5,6}.

NOH

Abbreviations: Cp, N⁴-hydroxycytidine 3'-phosphate; hydroxylamine-adduct of cytidine or C* is short for 5,6-dihydro-N⁴-hydroxy-6-hydroxylaminocytidine; CMC, N-cyclohexyl-N'-(4-methylmorpholinium) ethylcarbodiimide. For abbreviations of the minor components of tRNA, the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature have been followed: s⁴U, 4-thiouridine; m⁷G, 7-methylguanosine; Cm, 2'-O-methylcytidine.

- Bochkareva, E.S., Budker, V.G., Girshovich, A.S., Knorre, D.G., Teplova, N.M. (1973) Mol. Biol. 7, 278-288

Volume 2 number 8 August 1975

Nucleic Acids Research

Fluorescence probing of nucleic acids: I. singly and doubly labeled dithymidine phosphate: fluorescence and energy transfer studies

Jacques Tournon

Groupe de Physique des Solides de l'Ecole Normale Supérieure, Tour 23,
2 Place Jussieu, Paris 5e, France

Received 25 April 1975

ABSTRACT

Dithymidine phosphate labeled at its 5' end with a naphthalene-carbamate characterized by chromatography and absorption and fluorescence measurements. Models for three possible conformers where the dye is solvated, stacked on the first thymine or intercalated between the two thymines are given with their absorption and fluorescence spectra. The doubly labeled molecule, stacked on the D and A form a donor-acceptor energy transfer pair has also been prepared. The energy transfer rate has been measured from the donor fluorescence lifetime.

INTRODUCTION

Fluorescence probing has proved to be a powerful technique to study conformations of biomolecules and to investigate interactions involving biopolymers. Although many studies have been reported of fluorescence probing of protein conformations, only few studies have dealt with fluorescence probing of nucleic acids conformations¹. The difficulty in probing nucleic acids lies in their structure; there is no unique site for interaction of a fluorescent dye with nucleic acids in contrast with a protein where a probe often interacts with the active site. In addition aromatic amino-acids, particularly tryptophan, can act as intrinsic probes of protein fluorescence. One approach to tackle the problem is to prepare fluorescent base analogues². Our approach is to label nucleic acids with dyes at specific sites, for example the 3' and 5' positions of the end riboses of the chain. Such singly labeled nucleic acids can be used to study proteins-nucleic acids interactions. One may also prepare a doubly labeled nucleic acid and use the efficiency of energy transfer to study conformational changes due to its interaction with another nucleic acid chain or a protein. Obviously this technique is limited to short labeled DNA chains since energy transfer can hardly be measured beyond 50 Å.

In this paper we shall discuss the preparation and spectroscopic

properties of a dithymidine monophosphate which is labeled at its 5' end with a naphthalene derivative and at its 3' end with an anthracene derivative, forming a donor-acceptor pair (DTpTA). The energy transfer rate in this doubly labeled nucleic acid is obtained from fluorescence lifetime measurements. Implications of these results for the determination of the chain conformations are discussed.

I - CHEMICAL SYNTHESIS

The method used to synthesize the doubly labeled dithymidine phosphate DTpTA is applicable to longer chains; it consists of three steps:

- Preparation of the 5' labeled monomer (DT)

Preparation of 5' naphthalene-2-carbamate thymidine-3'OH (5'NC-Thy-3'OH) compound II in Fig. 1 was described in an earlier paper³. It is prepared by the reaction of 2-naphthalene isocyanate on thymidine protected at its 3' position.

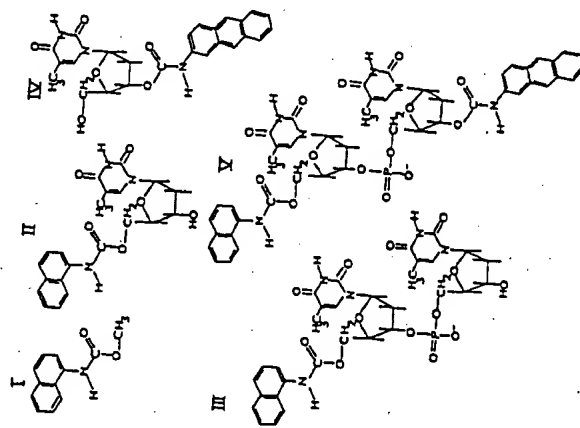


Fig. 1 : Compounds referred to in the text.

b) Condensation (DTpT)

An excess of the 5' labeled nucleotide is condensed with 5'-monophosphate-thymidine to give the dimer. The condensation is done by the procedure used for oligothymidic acids⁴ in dry pyridine with dicyclohexyl-

carbodiimide as the condensing agent. To avoid condensation of non-labeled nucleotides we used a 3' acetyl protected phosphate thymidine. The condensation reaction is written as follows :



The condensed product can be deacetylated easily⁴ without removing the 5' label in ammoniacal solution at pH = 11.

Separation of the condensation products was done on a DEAE-cellulose column, ammonium acetate was used as eluent. The elution pattern is shown on Fig. 2a. Three main peaks are observed : A, C, D. Thin layer chromatography (TLC) (on silicagel sheets with eluent : 80% acetonitrile - 20% water) shows that product A is 5'-naphthalene carbamate-thymidine ($R_f = 0.94$) and that products C ($R_f = 0.73$) and D ($R_f = 0.65$) are new compounds.

C is four times more abundant than D. The nature of products C and D was checked by enzymatic hydrolysis with snake venom phosphodiesterase, which gives back the two starting compounds, i.e. 5'-naphthalene-2 carbamate thymidine-3'OH (compound II of Fig. 1) and 5'-phosphate-thymidine-3'OH, identified by TLC. These two hydrolysis products were separated on DEAE-cellulose columns and their quantities determined by absorption spectroscopy. Both compounds C and D were found to contain equal amounts of the two hydrolysis products. From this analysis two conclusions can be drawn :

- C and D are both condensation products of one 5'-Naphthalene carbamate-thymidine and one 5'-phosphate thymidine.

- In both compounds condensation is through a O-P-O bridge, since this is the basic requirement for phosphodiesterase action⁵. These results can be taken as evidence that both products C and D correspond to compound III, Fig. 1, they will be called IIIC and IIID.

c) Labeling at the 3' end (DTpTA)

Labeling of the 3'OH end of compound IIIC was done by action of anthracene-2-isocyanate in dry pyridine at room temperature. Anthracene-2-isocyanate is not commercially available and is prepared by action of phosgene on 2-anthracene⁶. Excess of anthracene isocyanate after completion of the reaction is removed by addition of methanol to give 2-anthracene-methyl carbamate. The product is separated on a DEAE-cellulose column and the elution diagram is shown on Fig. 2b. The first eluting product is shown by TLC to be anthracene-methyl carbamate. The second product is shown by comparison of its absorption and fluorescence spectra to those of IV (see next paragraph) to contain Anthracene carbamate, it is considered as product V, Fig. 1.

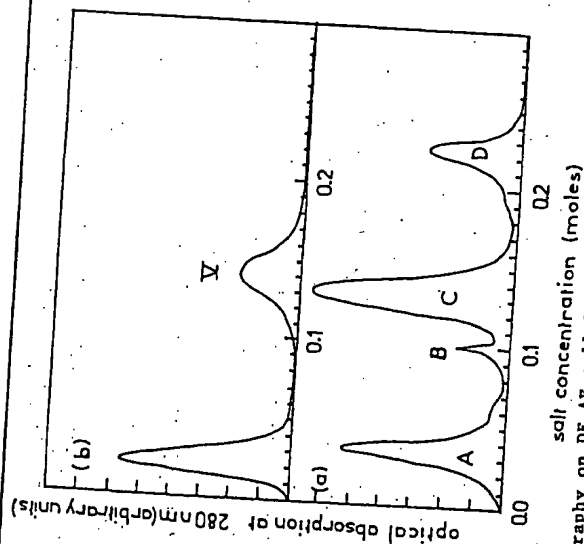


Fig. 2: Chromatography on DE AE cellulose column.

a) Elution diagram of compounds after condensation (I b). B produced in small amount does not contain naphthalene, it is not studied further.
b) Elution diagram after labeling the 3' end (I c).

In addition, 5'OH thymidine-3'anthracene carbamate (IV on Fig. 1) was prepared by direct action of 2-anthracene isocyanate on 5'-trityl-thymidine 3'OH and subsequent detritylation as described for 5'OH-thymidine-3'naphthalene³. The product was analysed by NMR and shown to contain both anthracene and thymine. Compound IV was used as a reference for absorption and fluorescence studies.

II - OPTICAL MEASUREMENTS

Absorption spectra were run on a Beckman Acta III spectrometer, fluorescence spectra on a Jobin-Yvon spectrofluorimeter. Fluorescence spectra were not corrected for the wavelength dependent response of the instrument. Lifetime measurements were done with an instrument built on single photon counting techniques. The excitation light is produced at a repetitive rate of 60 000 per second by a spark in air; it has a width of 2.5 ns. Optical filters are used for excitation and emission lights: for the donor fluorescence a MTO 308 in excitation and a Kodak Wratten VOM 26 in emission, for the acceptor fluorescence a MTO 404 for excitation and a VOM 22

for emission. Excitation light was convoluted with different exponentials to fit the fluorescence decay curves. Details of the set up will be given in a later publication by M. Lebret and J.M. Le Pecq.

III - RESULTS AND DISCUSSION

a) Study of compound II

Absorption and fluorescence, spectra and fluorescence quantum yield of II in methanol have already been reported³. From methanol to water while negligible change in absorption is observed, the maximum of the emission band shifts from 350 nm to 375 nm (Fig. 3). The fluorescence yields and

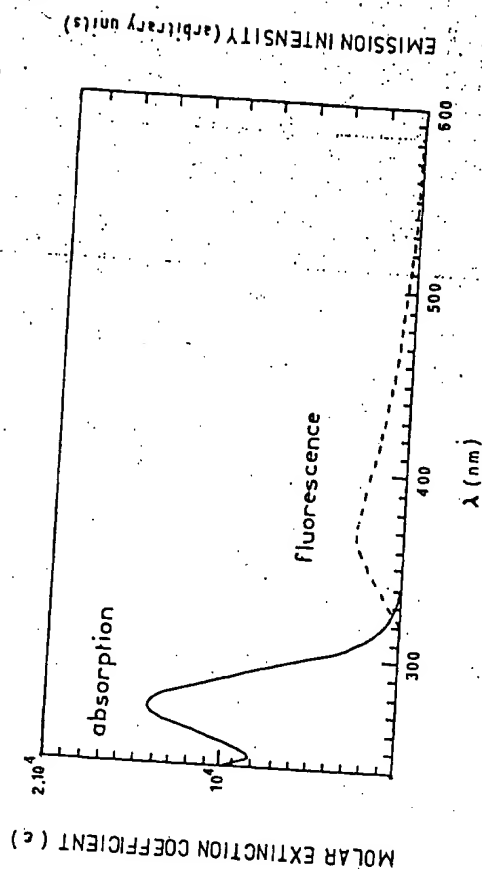


Fig. 3: Absorption and fluorescence spectra of compound II in water at room temperature.

lifetimes of II in methanol and water are shown on Table I. For comparison we have tabulated the fluorescence yields and lifetimes of the isomer of II, i.e. 5'OH-thymidine-3'α-naphthylcarbamate.

The data from Table I lead to the following observations:

- 1) Thymine drastically quenches the fluorescence of the dye when it is bound to the 5' position of thymidine but not when bound to the 3' position.
- 2) This quenching effect is much larger in water than in methanol.

Molecular models show that in thymidine dye-thymine stacking can occur when the dye is at the 5' position (Fig. 4a) but not at the 3' position.

Compound	Q _F		τ _{nscc}	
	water	methanol	water	methanol
I	1.00	0.75	18	10
II	0.05	0.20	18	10
II _{iso}	0.85	0.75	18	10
III C	0.02		18	
IV	0.09		16	
donor			3	
acceptor			16	

Table 1 : Fluorescence quantum yields (Q_F) and lifetimes (τ_F) at room temperature in water and methanol. The yields were measured by comparison with naphthalene in cyclohexane for which Q_F = 0.1 is taken 10 and corrections were made for difference in refractive indices of water, methanol and cyclohexane. II_{iso} = 5'-OH-thymidine-3'-α naphthalene carbamate

It has been shown⁷ that stacking can produce quenching of fluorescent molecules by enhancement of their intersystem crossing rate due to charge-transfer interaction. On the other hand, stacking in polynucleotides is stronger in water than in organic solvents such as methanol. These remarks suggest that fluorescence quenching in II is caused by a dye-thymine stacking interaction. Table I shows in addition that fluorescence lifetimes for compounds I and II are the same in a given solvent in spite of very different quantum yields. This striking result can be understood if we consider the following model :

- 1) The dye in II can be in two states, in the first the dye is not stacked with thymine (Fig. 4b) and has the same fluorescence yield as in I, in the second state the dye is stacked with thymine (Fig. 4a) and its fluorescence is completely quenched.
- 2) The dye cannot pass from one state to the other during the fluorescence lifetime.

These two states can be two conformations, or two sets of conformations of molecule II. Quantum yield values show that in water, fluorescent non-stacked molecules represent only 5% of all molecules II.

b) Study of compounds C and D

Results of enzymatic analysis given above show that the two fractions C and D correspond to the same structure III of Fig. 1. Absorption spectra of C and D are closely related, the maximum of the first absorption band of C is at 268 nm, while that of D is at 260 nm.

The fluorescence spectrum of III C (Fig. 5) has its maximum at 375 nm like those of I and II. Its fluorescence quantum yield is still smaller than that of II, while its fluorescence lifetime is still smaller results indicate along the model given in paragraph IIIa that the conformations where the dye is stacked on the neighbouring thymine (Fig. 4a) is still more favoured for III C than it is for II, and that the molecules for which the dye is not stacked (Fig. 4b) represent in this case only 2% of all molecules present.

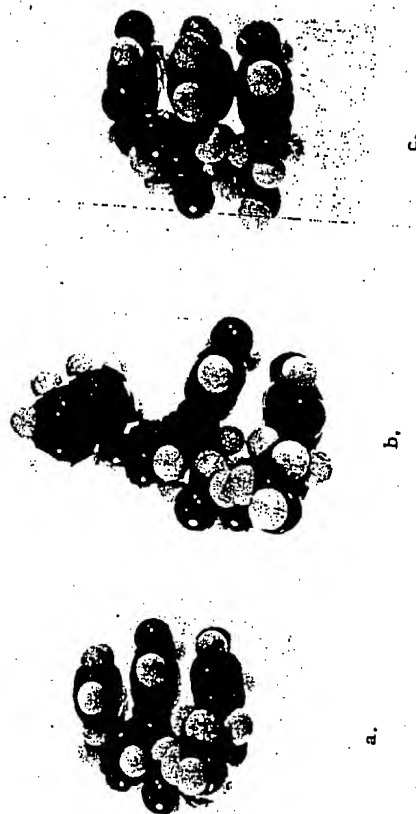


Fig. 4 - a - molecules C in "stacked" conformation
- b - molecules C in "non-stacked" conformation
- c - molecules C in "intercalated" conformation.

The fluorescence spectrum of compound III D is considerably red-shifted compared to those of I, II and III C, its maximum being at around 435 nm (Fig. 5). If as stated above III C and III D correspond to the same structure they should be conformational isomers (or conformers). These conformers are stable at room temperature in water and in the conditions of chromatography on cellulose. We looked for pathways from one conformer to the other, the conversion being monitored by the fluorescence spectra. The stability of conformers III C and III D seems quite high since heating to 90°C in water

proved insufficient. It is well known that large concentrations of urea can destroy tertiary structure in nucleic acids. The presence of urea to concentrations up to 7 M is insufficient at room temperature but at 90°C it can induce transition from IIIC to IIID (fig. 5) but not from III D to III C, indicating that conformer III D is more stable than conformer III C.

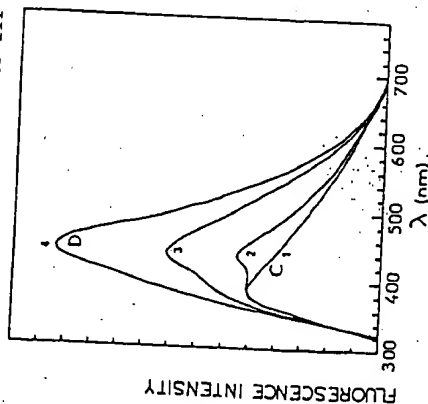


Fig. 5 : Fluorescence spectra of conformers IIIC and IIID and transition from IIIC to IIID monitored by fluorescence spectra after heating in 7 M urea at 90°C
1 - Conformer IIIC
2 - After heating 20 min
3 - After heating 1 h 30
4 - After heating 5 h, nearly complete conversion to IIID
An isostilbic point appears at 340 nm

Observation of molecular models shows that the naphthalene dye is bound to thymidine with enough flexibility so that it can pass under the neighbouring thymine. This leads us to a tentative model for structure of conformer IIID where the naphthalene dye is intercalated between the two thymines (Fig. 4c). The intercalation could explain the fluorescence spectrum of IIID at 435 nm which is never observed with the isolated dye I, or the labeled monomer II. The hydrophobicity of the dye would make the intercalated conformer (Fig. 4c) more stable than the conformers in which the dye is solvated (Fig. 4b) or stacked with the neighbouring thymine (Fig. 4a). The dye cannot intercalate between the bases by a simple translation movement parallel to the bases but has to perform a translation plus a rotation which require that the two thymine separate by more than the thickness of the dye. This stretching of the

molecule in the transition state must be energetically disfavoured which explains the difficulty to pass from IIIC to IIID. During the formation of compound III the condensation of 5'-phosphate thymidine on compound II traps the dye in conformations where it is on one side or the other of thymine, which will correspond to conformers IIIC or IIID. This trapping would explain how conformer IIIC can be five times more abundant though less stable than conformer IIID.

c) Absorption and emission spectra of the energy acceptor in IV

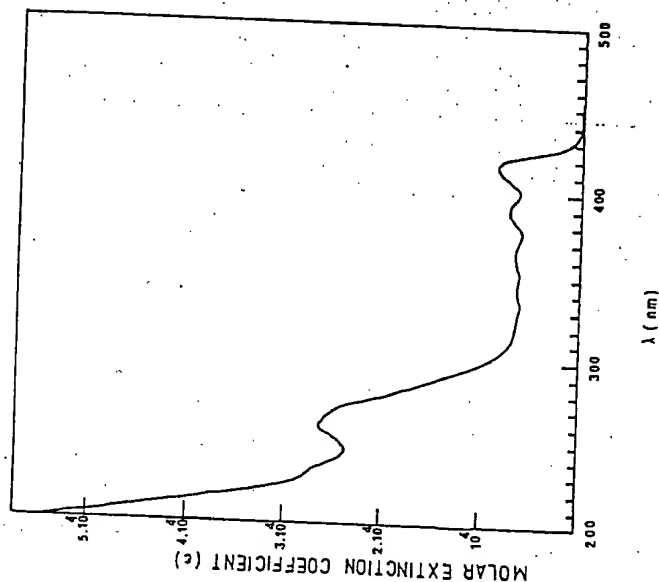


Fig. 6 : Absorption spectrum of compound IV in water at room temperature

The first and second absorption bands of the acceptor IV in water are shown in Fig. 6. excitation and emission spectra for fluorescence are shown in Fig. 7. The region of interest shows two excitation bands and an excitation minimum at 300 nm. The quantum yield of IV in water is 0.09; a solution of anthracene in cyclohexane was used as a reference. The fluorescence lifetime of IV was measured as 16 nsec.

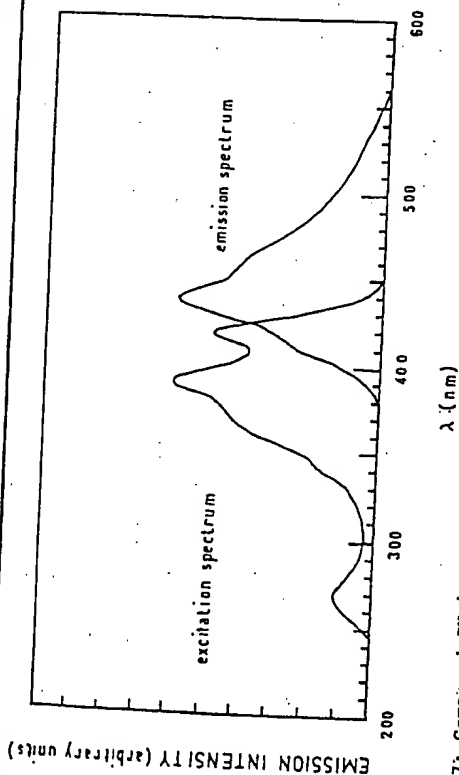


Fig. 7: Compound IV in water at room temperature:
 - fluorescence emission spectrum excited at 370 nm
 - excitation spectrum of the emission at 370 nm
 - excitation spectrum of the emission at 460 nm.

d) Energy transfer in the doubly labelled dimer V

The emission spectrum of V in water excited at 308 nm, (fig. 8), shows both donor and acceptor emissions. The excitation wavelength was chosen such that direct excitation of the acceptor moiety is at a minimum while the donor is efficiently excited. Evidence of energy transfer can be derived from a comparison of excitation spectra of the acceptor fluorescence in the absence (compound IV) or in the presence (compound V) of the donor moiety. It can also be derived from comparison of the donor fluorescence yields in III C and V. However these methods are not direct and they require a number of corrections:

- 1) Direct excitation of the acceptor must be corrected for.
- 2) Corrections must be made for absorbancies at 308 nm so that the fraction of light that is absorbed by the donor moiety alone is calculated.
- 3) Changes in quantum yield of the donor by the presence of the acceptor moiety may be induced by factors other than energy transfer, for example the acceptor moiety may induce changes of conformation of III C. This effects occurs in compounds II and III C, the presence at the second thymine in III C decreasing the donor fluorescence yield (Table I).

In contrast evaluation of energy-transfer from lifetime values is direct and does not require correction for absorption of the other moieties in the molecule. Moreover we have seen that the donor fluorescence lifetime remains constant in I, II, and III C and seems to be quite insensitive to stacking.

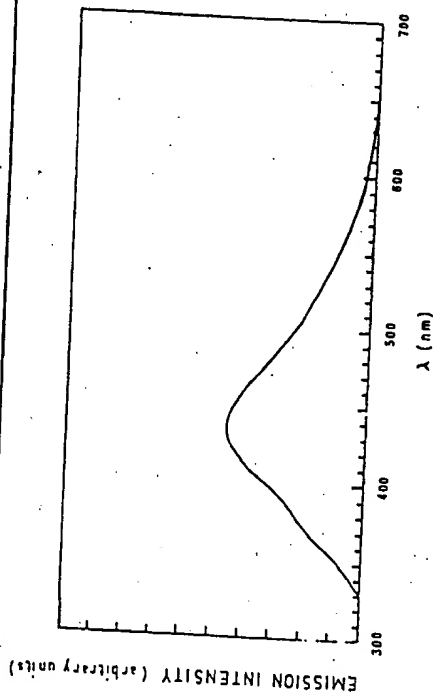


Fig. 8: Emission spectrum of V excited at 300 nm in water, at room temperature.

We have measured the donor fluorescence lifetime in V and compared it to that in III. A short lifetime component of 3 ns is observed which accounts for 99% of the emitted light (a second component of 16 ns is shown to be acceptor fluorescence passing through the emission filter). The drastic drop in the donor fluorescence lifetime is taken as evidence that a new deactivation process of the donor fluorescence is operating in V due to the presence of the acceptor dye. This deactivation process can be energy-transfer to the acceptor or can be induced by conformation changes in V due to the presence of the acceptor on the molecule. However this second hypothesis can be ruled out on the following grounds:

- 1) In I, II and III C donor fluorescence lifetime is the same in spite of the presence of the dithymidine phosphate and further addition of the acceptor separated from the donor by two thymidines is very unlikely to cause any serious perturbations on the latter.
- 2) The acceptor fluorescence excited at 400 nm has the same lifetime in molecules IV and V (Table I), which shows that the presence of the donor dye at one end of the dithymidine phosphate does not affect the acceptor fluorescence lifetime at the other end.

In conclusion, we consider the measured decrease in the donor fluorescence lifetime as a direct evidence for energy transfer to the acceptor in compound V. We call k_D the fluorescence rates of the donor in II and III C and k_A the energy transfer rate in V. From lifetime measurements of compounds III C and V (Table I):

$$\frac{1}{k_D} = 18 \text{ ns and } \frac{1}{k_D + k_T} = 3 \text{ ns} \quad \text{hence } \frac{k_T}{k_D} = 6$$

Forster's formula for the donor-acceptor energy transfer rate constant k_T can be written⁸ as a function of R :

$$k_T = k_D \left(\frac{R_0}{R} \right)^6 \dots \quad (1)$$

with

$$R_0^6 = 0.87 \times 10^{-24} \frac{Q_D K^2}{n^4} \quad (2)$$

where R_0 is the donor-acceptor distance at which k_T equals the donor fluorescence rate constant k_D . Q_D is the fluorescence quantum yield of the donor, i.e. the dye in non stacked conformations, i.e. $Q_D = 1$.

$I = \int_{\nu_1}^{\nu_2} \frac{f_D(\nu) \epsilon_A(\nu) d\nu}{\nu^4}$ is the overlap integral of donor fluorescence and

acceptor absorption, where ν is the wave number in cm^{-1}

distribution of donor fluorescence normalized such that $\int_{\nu_1}^{\nu_2} f_D(\nu) d\nu = 1$, $\epsilon_A(\nu)$ is the acceptor decadic extinction coefficient,

n is the refractive index of the solvent, in this case water. K is the orientation factor. $K = \cos \phi_{DA} - 3 \cos \phi_D \cos \phi_A$, where ϕ_{DA} is the angle between the transition moment vectors of both molecules, ϕ_D and ϕ_A are the angles between vector DA and D and A transition moment vectors.

If donor and acceptor chromophores are free to rotate at a rate faster than the transfer rate, the average value of K^2 is $2/3$, in this case the calculated value for R_0 using (2) is 32 \AA . From the donor fluorescence lifetime measurement we find $R = 24 \text{ \AA}$ from (1). This value is consistent with the structure given for pTPT⁹ and molecular models.

However there is no evidence that may support the assumption that the two dyes are free to rotate on the chain. On the contrary, this work has shown that naphthalene carbamate dyes bound in 5' position fluoresce and can act as donors in only a fraction of all molecules V present in solution. These correspond to conformers where the dye and the thymine are not stacked. These conformers are separated from stacked conformers by an energy barrier that cannot be overcome at room temperature during the donor fluorescence lifetime of 18 ns . Hence the possible directions that donor transition

moments can assume on the chain may be limited. On the 3'-end of molecule V the anthracene carbamate acceptor dye is seriously limited in its rotation by the steric hindrance of the ribose ring to which it is directly bound.

In conclusion, this work on TPT labelling shows that oligo-nucleotides can be labelled with fluorescent dyes specifically at both ends. This specificity gives to such systems a potential use to study their conformational changes due to interaction with proteins and nucleic acids. The relatively long lifetime value for the fluorescent donor and the accuracy in fluorescence lifetime measurements allow determination of energy transfer rates for longer oligo-nucleotides containing up to 10 residues.

ACKNOWLEDGEMENTS

The author wishes to thank Dr M. LEBRET with whom fluorescence lifetime measurements have been made on his equipment, Dr A. BERNARDI for his help in the enzymatic analyses, Dr SPACH, Mme CAILLE, M. BOUTROY for their help in the preparation of anthracene isocyanate, and Drs EL-BAYOUMI, LANG, SCHOTT, G. BERNARDI, A.M. MICHELSON, for fruitful discussions.

REFERENCES

1. Pochon F., Kapuler A.M., Michelson A.M. (1971) *Proc. Nat. Ac. Sc. US* **68**, 205
2. Chih-hsin Yang, Soll D., (1974) *Biochemistry* **13**, 3615
3. Cantor C.R., Tao T., (1971) *Proc. Nucleic Acid Res.* **2**, 31
4. Ward D.C., Reich E., Stryer L. (1969) *Biochemistry* **24**, 1228
5. Secrist J.A. et al., (1972) *Science* **177**, 279
6. Bienvenue A., Tournon J. (1973) *Biochimie* **55**, 1167
7. Khorana H.G., Connors W.J., (1965-1969), *Biochemical Preparations* **113-12**.
8. Razzell W.E., Khorana H.G., (1959), *J. Biol. Chem.* **234**, 2105
9. Fieser L.F., Creech H.J. (1939) *J. Am. Chem. Soc.* **61**, 3502
10. Majumdar D.K., Basu S., (1960) *J. Chem. Phys.* **33**, 1199
11. Tournon J., El-Bayoumi M.A., (1972) *J. Chem. Phys.* **56**, 5128
12. Forster T., Kuntz E., El-Bayoumi M.A., (1972) *Photochem. Photobiol.* **16**, 425
13. Birks J.B. (1969) *Photophysics of Aromatic Molecules*, Wiley, Interscience

METHODS IN ENZYMOLOGY⁷

EDITORS-IN-CHIEF

Sidney P. Colowick Nathan O. Kaplan

Methods in Enzymology

Volume XL

Hormone Action

Part E

Nuclear Structure and Function

EDITED BY

Bert W. O'Malley

DEPARTMENT OF CELL BIOLOGY
BAYLOR COLLEGE OF MEDICINE
TEXAS MEDICAL CENTER
HOUSTON, TEXAS

Joel G. Hardman

DEPARTMENT OF PHYSIOLOGY
VANDERBILT UNIVERSITY SCHOOL OF MEDICINE
NASHVILLE, TENNESSEE

1975



ACADEMIC PRESS New York San Francisco London
A Subsidiary of Harcourt Brace Jovanovich, Publishers

Sample Preparation. Most of the important considerations of sample preparation for liquid scintillation counting can be conveniently reviewed in references cited in footnotes 15 and 16. It is worth reiterating that for calculations of the radioactivity of an isotope pair to be reliable, both isotopes must be uniformly distributed in the sample. Translucent emulsions with microscopic micelles may be counted as well as true solutions if three cautions are observed: (a) If one of the isotope pair is ^3H or ^{125}I , it must be in the same phase as the other isotope. This might seem to be a trivial problem since emulsion counting is of aqueous samples, but it is not; there may be differential extraction of the labeled solutes into the organic solvent. (b) Absorption (of the lower energy isotope in particular) onto the vial surface may greatly alter detection efficiency.¹⁷ (c) If the E_{max} of an isotope is equivalent to or greater than ^{14}C , and a commercial solubilizer of aqueous samples has been included in the sample, the surfactant itself may fluoresce in response to radioactivity. This renders quench correction curves derived from sealed commercial standards invalid.¹⁸ All these proscriptions may be summarized in the cardinal, and frequently neglected, general rule for scintillation counting: standards should be of the same geometry as the unknown samples.

- ¹⁷ J. C. Turner, "Sample Preparation for Liquid Scintillation Counting," Review 6. The Radiochemical Centre, Amersham, England 1967. Reprinted in "Handbook of Radioactive Nuclides" (Y. Wang, ed.) pp. 256-273. Chem. Rubber Publ. Co., Cleveland, Ohio, 1969.
- ¹⁸ "The Current Status of Liquid Scintillation Counting" (E. D. Bransome, ed.), Chapters 16-27. Grune & Stratton, New York, 1970.
- ¹⁹ G. J. Litt and H. Carter, in "The Current Status of Liquid Scintillation Counting" (E. D. Bransome, ed.), pp. 156-163. Grune & Stratton, New York, 1970.
- ²⁰ S. E. Sharpe, III and E. D. Bransome, Jr., *Anal. Biochem.* 56, 313 (1973).

[22] Use of Antibodies to Nucleosides and Nucleotides in Studies of Nucleic Acids in Cells

By B. F. ERLANGER, W. J. KLEIN, JR., V. G. DEV, R. R. SCHRECK, and O. J. MILLER

The preparation of purine- and pyrimidine-protein conjugates and their use in eliciting base-specific antibodies that react with nucleic acids have been described in this series, Volume 12B [173]. Their reaction with nucleic acids requires that the purine or pyrimidine bases be unpaired, i.e., that the nucleic acid be denatured or, at least, have single-stranded regions. It follows, therefore, that demonstrable reaction with these antibodies is evidence for "single-stranded areas." Fluorescein-tagged

antibodies have been used to study cell nuclei^{1,2} and metaphase chromosomes.²⁻⁴ With respect to the former, it was found that single strandedness could be demonstrated only during S phase of the cell cycle.^{1,2} In studies with metaphase chromosomes, local areas, rich in A-T and G-C base pairs, have been localized.

Nuclear Fluorescence of Mouse L Cells

Fluorescein Conjugates. Fluorescent sheep anti-rabbit globulin and fluorescent rabbit anti-sheep globulin were obtained from Pentex Co., Kankakee, Illinois and from Nutritional Biochemicals Corp., Cleveland, Ohio. Using antinucleoside or anti-BSA antisera, globulin preparation and purification of the fluoresceinated conjugates were carried out as described by Dedmon, Holmes, and Deinhardt.⁵ Crystalline fluorescein isothiocyanate (Sylvania Chemical Co., Orange, New Jersey) was conjugated to protein following the procedure of Hsu (K. Hsu, personal communication). After each step in the preparation and purification of conjugates, the antisera were checked for the presence of antibody by the gel diffusion method.

Tissue Culture. Mouse L cells (derived from strain 929, Earle) were grown in 250-ml plastic tissue culture flasks (Falcon Plastic Co., Los Angeles, California) at 37° for 4-7 days. The sheet was detached and dispersed in 0.25% trypsin in phosphate-buffered saline (PBS) with sodium ethylenediamine tetraacetate and glucose.⁶

The cells were collected by centrifugation and suspended in culture medium. (Eagle's minimum essential medium⁷ with 10% fetal calf serum, heat treated at 56° for 30 minutes to inactivate possible mycoplasma contaminants; penicillin G, 15 units/ml; streptomycin, 15 µg/ml; and the concentration of glutamine, cofactors, and vitamins; and nonessential amino acids at 0.1 mM: L-alanine, L-asparagine, H₂O, L-aspartic acid, L-glutamic acid, L-proline, L-serine, and glycine. All medium constituents were obtained from Grand Island Biological Co., Grand Island, New York).

- ¹ W. J. Klein, Jr., S. M. Beiser, and B. F. Erlanger, *J. Exp. Med.* 125, 61 (1967).
- ² M. V. R. Freeman, S. M. Beiser, B. F. Erlanger, and O. J. Miller, *Exp. Cell Res.* 69, 345 (1971).
- ³ V. G. Dev, D. Warburton, O. J. Miller, D. A. Miller, B. F. Erlanger, and S. M. Beiser, *Exp. Cell Res.* 74, 288 (1972).
- ⁴ R. R. Schreck, D. Warburton, O. J. Miller, S. M. Beiser, and B. F. Erlanger, *Proc. Nat. Acad. Sci. U.S.A.* 70, 804 (1973).
- ⁵ R. E. Dedmon, A. W. Holmes, and F. Deinhardt, *J. Bacteriol.* 89, 734 (1965).
- ⁶ J. Paul, "Cell and Tissue Culture," 3rd ed. Williams & Wilkins, Baltimore, Maryland, 1965.
- ⁷ H. Eagle, *Science* 130, 432 (1959).

Plastic flasks with 25 ml of culture medium were inoculated with 4×10^6 cells (approximately) or Leighton tubes containing cover slips were inoculated with 1.3 to 1.5×10^5 cells in 1 ml of medium. The cover slips were removed after various times of incubation and, after three washes of 5 minutes each in phosphate-buffered saline (PBS) ($0.14 M$ NaCl, $10 mM$ PO₄, pH 7.3), were fixed by one of the following methods: (a) air-drying 1–2 hours at 37° ; (b) air-drying 1–2 hours at 37° , storing at 4° for 1–21 days, and dipping in methanol at room temperature immediately prior to staining; and (c) air-drying 1–2 hours at 37° , placing at 4° for 1–21 days followed by treatment with 95% ethanol for 30 seconds at room temperature just prior to staining. Method (c) gave the most consistent results.

Fluorescent Antibody Staining. Antisera were diluted in PBS to 2–4 mg/ml total protein as measured by a hand refractometer. The coverslips were placed on a small staining rack in a humidity chamber, and 5 drops of antiserum were added onto the surface of each coverslip. The antiserum was allowed to react for 20 or 30 minutes at room temperature. Then the coverslips were drained and rinsed with a forced stream of PBS. This was followed by 2 washes in PBS for 10 and 5 minutes duration and another vigorous rinsing with PBS. The coverslips were then mounted in glycerol (1 part) : PBS (4 parts) pH 7.5 on standard microscope slides (less than 1 mm thick) and immediately examined under the UV microscope.

In "blocking" experiments, unfluoresceinated globulin was allowed to react with the cells as above for 1 hour (1 replacement with fresh globulin at 0.5 hr) at 37° . The coverslips were washed as above and then stained with the fluoresceinated antiserum.

Microscopy and Photography. A Zeiss Standard Universal Microscope fitted with an HBO 200 Osram mercury burner and a $1.2/1.4$ Z dark-field condenser was employed for UV microscopy. The exciter filters were BG 12, 4 mm, and BG 39, 2.5 mm. Barrier filters were Zeiss 47 or 50. The lens system consisted of a $40 \times$ oil immersion objective, a $10 \times$ eyepiece ($5 \times$ eyepiece to camera) and a $2 \times$ Optovar. Photographs were taken with a Zeiss Ikon 35mm camera (factor 0.5) using Anscochrome 200 ASA color daylight or Agfa Isopan Record (black and white) film.

Reaction of Anti-nucleoside Antibodies with Human Metaphase Chromosomes

The anti-nucleoside antibodies have been shown to bind to fixed human metaphase chromosomes only after treatment with denaturing agents such as aqueous solutions of NaOH.² Such treatment can produce swollen

and distorted chromosomes which obscure the pattern of antibody binding to chromosomes. We therefore investigated the possibility of replacing NaOH with a saline solution of formamide, which reduces the thermal stability of DNA.³ This method, described below, enabled the antinucleoside antibodies to bind to human chromosomes with minimal distortion.³ We then investigated other methods for generating single-stranded DNA in chromosomes. Methylene blue-mediated photooxidation selectively destroys guanine residues in DNA in solution,⁴ freeing the formerly hydrogen-bonded cytosines. We have used this procedure to attach anti-cytosine antibodies to fixed metaphase chromosomes.⁴

Preparation of Metaphase Chromosomes. Chromosome preparations were obtained from cell cultures set up from peripheral blood samples. milliliters of venous blood was collected in a syringe containing 0.5 ml of an isotonic aqueous solution of sodium heparin (Organon) to prevent clotting, and allowed to sit undisturbed at room temperature until the red blood cells have settled. About 1 ml of the resulting suspension of leukocytes in plasma was transferred to sterile tissue culture flasks containing 10 ml of complete Eagle's minimal essential media (MEM) (plus penicillin-streptomycin, L-glutamine and 20% fetal calf serum) and 0.5 ml of phytohemagglutinin "M" (General Biochemicals). Cultures were grown at 37° for 3 days. After this incubation, 0.2 ml of Colcemid ($10 \mu\text{g/ml}$) in Hanks' balanced salt solution (Grand Island Biological Co.) was added to each flask, which was then reincubated at 37° for 1 hour. The culture was centrifuged (800 rpm for 8 minutes), and the pellet was suspended in hypotonic solution ($0.075 M$ KCl). After 2 minutes, the suspension was centrifuged (800 rpm, for 8 min) and the pellet resuspended in fixative (3:1 methanol-glacial acetic acid). After three changes of fixative, slides were prepared. However, for photooxidation it proved advantageous to store the cells in fixative for several days before making slides. Slides were made by suspending the cells in fresh fixative and dropping the suspension from a Pasteur pipette onto cold, wet slides which were then air-dried. The slides were stored in the refrigerator until needed.

Chromosome preparations were also obtained from other types of cultured cells, growing either as suspension cultures or monolayers. The procedure is basically the same as for cultured leukocytes, except that cells growing attached to the glass or plastic culture vessel were first separated from the surface. Some dividing cells could be shaken off the surface of the vessel rather easily, and we prefer this method when it works. In

³ B. L. McCaughy, C. D. Laird, and B. J. McCarthy, *Biochemistry* 8, 3289 (1969).

⁴ A. G. Garro, B. F. Emlinger, and S. M. Beiser, in "Nucleic Acids in Immunology" (O. J. Plescia and W. Braun eds.), pp. 47–57, Springer-Verlag, Berlin and New York, 1968.

other cases, the culture medium was removed, a trypsin-EDTA mixture (0.5 g of trypsin, 1:250, and 0.2 g of EDTA per liter of Puck's saline A which consists of 8000 mg of NaCl, 400 mg of KCl, 1000 mg of glucose, 350 mg of NaHCO_3 , and 5 mg of phenol red per liter) was added and the cells were incubated at room temperature for a few minutes or until the cells could be removed by vigorous shaking of the vessel. The mixture was centrifuged as above, the cells were suspended in hypotonic solution (in this case 38 mM KCl). The remainder of the procedure is as already described.

Method of Denaturation

1. Formamide

Reagents

Formamide (Stabilized, Fisher Scientific Co.) pH adjusted to 7.2 with concentrated HCl

20 × SSC: 3 M sodium chloride and 0.3 M trisodium citrate

95% Formamide: 95 ml of pH 7.2 formamide + 5 ml of 20 × SSC Ethanol, 70%, 95%, absolute

Phosphate-buffered saline (PBS): 20 g of NaCl, 85 ml of 0.25 M NaH_2PO_4 , 15 ml of 0.25 M KH_2PO_4 , in 2400 ml of distilled water pH 7.2-7.4

Coplin jars designed to hold microscope slides were filled with 95% formamide in SSC and placed in a water bath maintained at 65°. The slides were heated for 1 hour in formamide. They were then rinsed twice in 70% ethanol, once in 95% ethanol and once in absolute ethanol. The slides were rehydrated for about 5 minutes in PBS before being treated with antibody.

2. Photooxidation

Reagents

Tris-HCl buffer, 0.1 M: dissolve 6 g of THAM (Fisher) in 500 ml of distilled water and adjust pH to 8.75 with concentrated HCl

Stock methylene blue (National Aniline): dissolve 0.0125 g of powdered methylene blue in 10 ml of Tris-HCl buffer

Methylene blue, 33.4 μM : dilute 0.5 ml of stock solution in 49.5 ml of cold Tris buffer

Slides were photooxidized in a Coplin jar containing a cold saturated solution of 33.4 μM methylene blue. Oxygen was bubbled into the jar,

which contained the dye solution and the slides, for 10 minutes through a Pasteur pipette. As oxygen is critical to the reaction, it is imperative to seal the jar tightly after the addition of oxygen. The sealed jar was placed in a glass water bath (25°). The jar was illuminated overnight (15-18 hours) through the water bath by a 150 W Sylvia flood lamp, which was 15 cm from the jar. As the reaction progressed, the dye solution became paler and was almost colorless after 18 hours. The final temperature within the Coplin jar was usually about 1° higher than that of the water bath. The slides were rinsed briefly in PBS before antibody treatment.

Indirect Immunofluorescence. Slides treated to produce denatured collagen were first layered with rabbit anti-nucleoside antisera diluted 1:1 with PBS and incubated at room temperature in a moist chamber for 45 minutes. Unbound antibody was rinsed off the slides with 200 ml of PBS from a spray bottle. Indirect immunofluorescence was accomplished by incubating the slides as before with a 1:50 dilution of sheep anti-rabbit IgG tagged with fluorescein. After a second washing, the slides (cell surface) were wetted with PBS, a clean coverslip was mounted, the excess buffer blotted off and the edges sealed with clear nail polish.

Microscopy and Photography. The slides were examined with a Zeiss fluorescent microscope using light from an HBO 200 W mercury lamp, transmitted through a cardioid condenser, with a BG 12 (4 mm) exciter filter, a 530 nm barrier filter and a 100 × Planapochromatic objective. Well spread metaphases were photographed on either Panatomic X or on H & W control film with 2-minute exposures.

Panatomic X film was developed with Microdol X (Kodak), and H & W film was processed with H & W control developer (H & W, St. Johnsbury, Vermont). The negatives were printed on Ilford paper, Grade 4.

[23] Techniques for the Study of Hormone Effect on Collagens

By DOROTHY H. HENNEMAN and GEORGE NICHOLS, JR.

I. General Precautions	309
II. Initial Preparation of Tissues	310
A. General	310
B. Specific Tissues	311
C. Defatting, Drying, and Demineralization	312
D. Methods to Prepare Suspensions of Isolated Cells	312

Synthesis and Properties of Fluorescent Nucleotide Substrates for DNA-dependent RNA Polymerases*

(Received for publication, July 13, 1979)

L. R. Yarbrough,† Joseph G. Schlageck, and Michael Baughman

From the Department of Biochemistry, University of Kansas College of Health Sciences and Hospital, Kansas City, Kansas 66103

A new class of fluorescent nucleotide analogs which contain the fluorophore 1-aminonaphthalene-5-sulfonate attached via a γ -phosphoamidate bond has been synthesized. Both the purine and pyrimidine analogs have fluorescence emission maxima at 460 nm. Cleavage of the α - β -phosphoryl bond produces change in both the absorption and fluorescence emission spectra. The fluorescence of the pyrimidine analogs is quenched; cleavage of the α - β -phosphoryl bond of the UTP analog produces about a 14-fold increase in fluorescence intensity at 500 nm. Under the same conditions the fluorescence of the CTP analog increases about 8-fold, whereas the fluorescence of the purine analogs shows only a slight change. These derivatives are good substrates for *Escherichia coli* RNA polymerase with only slightly increased K_m values and with V_{max} values about 50 to 70% that of the normal nucleotides. They are used less efficiently by wheat germ RNA polymerase II. The ATP analog can be used by *E. coli* RNA polymerase to initiate RNA chains.

Nucleotides play an important role in many metabolic processes. These include DNA, RNA, and protein synthesis, assembly of structural proteins such as tubulin, and energy metabolism. A variety of nucleotide analogs have been synthesized which have proven extremely useful in obtaining information about such processes. These include those with altered phosphoryl structures such as AMP-PNP (1, 2) and ATP- γ -S (3), analogs with modified ribose rings (4, 5), and others having altered purine or pyrimidine ring structures such as ϵ -ATP (6) and S^6 -GTP (7).¹

Several fluorescent nucleotide analogs have been synthesized. These include ϵ -ATP, formycin triphosphate, and 2-aminopurine triphosphate (8, 9), the lin-benzo ATP analogs (10, 11), and a fluorescent GTP analog (12). All of these derivatives have altered purine ring structures.

The DNA-dependent RNA polymerases synthesize RNA in

* This research supported by a grant to L. R. Y. from the National Foundation-March of Dimes. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed.

The abbreviations used are: AmNS, 1-aminonaphthalene-5-sulfonate; (γ -AmNS)ATP, adenosine-5'-triphospho- γ -1-(5-sulfonic acid)naphthylamide; (γ -AmNS)NTP, ribonucleoside-5'-triphosphate containing 1-aminonaphthalene-5-sulfonate attached via a γ -phosphoamidate bond; (AmNS)PP_i, the pyrophosphate adduct of 1-aminonaphthalene-5-sulfonate; ϵ -ATP, 1- N^6 -ethenoadenosine-5'-triphosphate; ATP- γ -S, adenosine-5'-O-(3-thiotriphosphate); AMP-PNP, adenylyl-5'-yl imidodiphosphate; TEA, triethylamine- HCO_3^- buffer; TKME buffer, 0.05 M Tris, pH 8, 0.05 M KCl, 10^{-2} M $MgCl_2$, 10^{-4} M EDTA; PEI, polyethyleneimine; SDS, sodium dodecyl sulfate.

the presence of a template, NTPs, and a divalent cation (see Chamberlin (13) for a review). The synthesis of an RNA chain involves at least the following events: 1) template binding, 2) binding of the first two NTPs, 3) formation of the first phosphodiester bond (initiation), 4) binding of subsequent NTPs and phosphodiester bond formation (elongation), and 5) RNA chain termination. In addition, there must be a translocation of the DNA and RNA with respect to the enzyme after each phosphodiester bond is formed. Although the template binding step has been studied in some detail, much less is known about subsequent events (steps 2 to 5).

In some elegant studies, Vallee and co-workers (14-16) have demonstrated that fluorescent substrates can be used to great advantage for studies of substrate binding and subsequent transformations. They synthesized a series of dansylated peptide substrates for carboxypeptidase and then studied the binding and hydrolysis (product release) steps by fluorescence stopped flow. When the dansylated peptides bound, they quenched intrinsic enzyme fluorescence due to resonance energy transfer; when the products were released, energy transfer was eliminated and the fluorescence increased. A similar approach could provide valuable information about the events involved in RNA synthesis. Such studies require fluorescent nucleotide analogs which have well characterized fluorescence properties and which are good substrates. Although several fluorescent nucleotides are available which could possibly be used for such studies, all have severe limitations. ϵ -ATP has excellent fluorescence properties; however, it is neither a substrate nor an inhibitor for *Escherichia coli* RNA polymerase (17, 18). Formycin triphosphate is a substrate for *E. coli* RNA polymerase (7, 8) but it cannot be used to initiate RNA chains. Moreover, its quantum yield is low ($Q = 0.05$). 2-Aminopurine triphosphate has a relatively high quantum yield (8) but it cannot form the hydrogen bonds (H bonds) normally formed by GTP. Since H bonding apparently plays a critical role in substrate binding, neither 2-aminopurine triphosphate nor the fluorescent GTP analog synthesized by Weigand and Kaleja (12) would be likely to serve as a good substrate for RNA polymerase (19).

Grachev and Zaychikov (20) and Babkina *et al.* (21) have described the synthesis of an ATP analog containing aniline attached to the terminal phosphate via a phosphoamidate bond. This analog is a good substrate for *E. coli* RNA polymerase. Moreover, it is apparently also used to initiate RNA chains. This suggested to us that it should be possible to prepare similar nucleotide derivatives containing the fluorophore, 1-aminonaphthalene-5-sulfonate (AmNS).² In this

² We have used the abbreviation used by Turner and Brand ((1968) *Biochemistry* 7, 3381-3387) for the aminonaphthalene sulfonates rather than the term ANS which is commonly used to refer to the noncovalent fluorescent probe, 8-anilinonaphthalene-1-sulfonate.

communication we describe the synthesis of such analogs and their spectroscopic and biological properties.

MATERIALS AND METHODS

Chemicals—The following chemicals were purchased from the sources listed in parentheses: ribonucleoside triphosphates and poly(d(A-T))-poly[d(A-T)] (P-L Biochemicals), [^3H]ATP and [^3H]GTP (New England Nuclear), [^3H]UTP (ICN Radiochemicals), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Pierce), and 1-aminonaphthalene-5-sulfonate (Tridom). For the early studies 1-aminonaphthalene-5-sulfonate was recrystallized from water; in subsequent experiments, the unrecrystallized material was used. Tris (ultrapure) was obtained from Schwarz/Mann. Other chemicals used were reagent grade.

Other Materials—Plastic-backed polyethyleneimine cellulose thin layer plates with fluorescent indicator were from EM Labs and plastic-backed cellulose thin layer plates were from Eastman. Venom phosphodiesterase was purified from crude venom of *Crotalus adamanteus* (Sigma) by incubation for 3 h at 37°C and pH 3.6. Following such treatment it showed no detectable ATPase activity.

Enzyme Purifications—*E. coli* DNA-dependent RNA polymerase was purified by the polyethyleneimine procedure of Burgess and Jendrisak (22). The specific activity of the enzyme ranged from 500 to 800 nmol of [^3H]GMP incorporated/mg of enzyme/10 min using native calf thymus DNA as template. Sigma content ranged from 60 to 80% of saturation as determined by SDS-gel electrophoresis. Wheat germ RNA polymerase II was purified through the DEAE-cellulose step described by Jendrisak and Burgess (23). The partially purified enzyme from the DEAE-cellulose column was pooled, precipitated with 1.5 volumes of neutralized $(\text{NH}_4)_2\text{SO}_4$, and diluted with 0.02 M Tris, pH 8, containing 0.1 mM EDTA, 0.2 mM dithiothreitol, 3 mM mercaptoethanol, and 20% glycerol until the conductivity was equal to that of the buffer containing 0.05 M $(\text{NH}_4)_2\text{SO}_4$. The enzyme (50 ml, ~160 mg) was applied to a 40-ml Sepharose 4B column containing 1 mg/ml of denatured calf thymus DNA attached covalently. The column was washed with buffer containing 0.02 M Tris, pH 8, 0.05 M $(\text{NH}_4)_2\text{SO}_4$, 0.1 mM EDTA, 3 mM mercaptoethanol, 0.2 mM dithiothreitol, and 20% glycerol until the A_{280} of the eluate dropped below 0.1. The enzyme was then eluted with the same buffer containing 0.25 M $(\text{NH}_4)_2\text{SO}_4$. Yields of 20 to 30 mg of purified enzyme having a specific activity with denatured calf thymus DNA of 400 to 500 nmol of [^3H]GMP incorporated per mg of enzyme per 10 min were obtained. Assay mixtures for RNA synthesis (0.1 ml) contained: 0.05 M Tris, pH 8, 0.05 M $(\text{NH}_4)_2\text{SO}_4$, 3 mM MnCl_2 , 4×10^{-4} M unlabeled NTPs, 4×10^{-4} M [^3H]UTP or [^3H]GTP (~10,000 cpm/nmol), 2 mM dithiothreitol, and 20 nmol of denatured calf thymus DNA. Reactions were incubated for 10 min at 37°C and then placed on ice; 0.1 ml of 0.1 M sodium pyrophosphate was added, and [^3H]RNA was precipitated with cold 5% trichloroacetic acid. The precipitates were collected on Whatman CF/A filters and counted in a liquid scintillation counter.

Spectroscopic Measurements—Corrected fluorescence excitation and emission spectra were obtained with a Perkin-Elmer MPF-44 recording fluorescence spectrophotometer equipped with a differential corrected spectra attachment. Samples had an absorbance of ≤ 0.05 to prevent significant inner filter effects. Unless otherwise noted, spectra were recorded with 10 nm excitation and emission band widths. For recording excitation spectra, an integral filter (No. 35) which transmits light of ≥ 350 nm was used to eliminate second order radiation. Quantum yields were determined using quinine sulfate in 0.1 N H_2SO_4 as standard with an assumed quantum yield of 0.55 (24). Where necessary (absorbance greater than ~0.002), the observed spectra were corrected for absorption of the exciting light. The value of 0.55 was used rather than the more recent value of 0.70 obtained by Scott *et al.* (25) to facilitate comparison with previously published data on the quantum yields of aminonaphthalene sulfonate and its derivatives. Absorption spectra were obtained with a Perkin-Elmer model 576 UV-visible recording spectrophotometer with a base-line correction accessory. The absorbance of samples was ≤ 2 to prevent errors due to stray light. A 1 nm band width was used for recording spectra. The temperature in all spectroscopic measurements was $25 \pm 0.5^\circ\text{C}$. All spectra shown were obtained with samples in 0.05 M Tris, pH 8, 0.05 M KCl, 0.01 M MgCl_2 , and 10^{-4} M EDTA (TKME buffer).

Synthesis and Purification of (γ -AmNS)ATP—1-Aminonaphthalene-5-sulfonate (447 mg) was added to 10 ml of H_2O , and the pH was adjusted to 5.8 with 1 N NaOH. Any insoluble material was removed by centrifugation, yielding a solution which was essentially saturated for this pH value (~0.18 to 0.2 M). Four milliliters of 12.5 mM ATP

(pH ~5.8) and 2 ml of 1-ethyl-3-(dimethylaminopropyl)carbodiimide were added to a reaction vessel maintained at 20°C. The reaction was initiated by adding 10 ml of the 1-aminonaphthalene-5-sulfonate solution and allowed to continue for 2.5 h. The pH was kept between 5.65 and 5.75 by the periodic addition of 0.1 N HCl. After 2.5 h, the reaction was diluted to 50 ml and made 0.05 M in triethylamine- HCO_3^- buffer (pH ~7.5). The reaction products were placed on a 50-ml DEAE-cellulose column which had been equilibrated with 0.05 M TEA; the column was washed with 100 ml of 0.05 M TEA and eluted with a 1000-ml gradient (0.05 \rightarrow 0.4 M TEA). Approximately 20-ml fractions were collected. Absorbance and fluorescence profiles of the fractions were obtained after appropriate dilution. The fluorescent analog eluted after the peak of unreacted ATP and showed a brilliant blue fluorescence. Peak fractions were pooled, taken to dryness by flash evaporation at 25°C, and redissolved in H_2O /methanol (70/30); the evaporation process was repeated until excess TEA was removed. The purified material was dissolved in 0.5 to 2 ml of water. Purity was assessed by thin layer chromatography as described previously (18). If traces of free 1-aminonaphthalene-5-sulfonate were detected, the purified fluorescent nucleotide was rechromatographed on a 15-ml DEAE-cellulose column using a 300-ml gradient of TEA (0.05 \rightarrow 0.4 M).

(AmNS)PP, was prepared by reacting sodium pyrophosphate with AmNS and the water-soluble carbodiimide under the conditions described above. It was purified by chromatography on DEAE-cellulose and shown to be homogeneous on the two thin layer systems described below. The purified compound was degraded by bacterial alkaline phosphatase to form free AmNS.

Digestion of (γ -AmNS)NTPs—Digestions with venom phosphodiesterase and bacterial alkaline phosphatase were performed as described previously (18).

Thin Layer Chromatography—Samples containing 5 to 50 nmol of nucleotide were spotted 2.5 cm from the ends of plates of cellulose or PEI-cellulose and air-dried. PEI-cellulose plates were developed by ascending chromatography for 6 cm in 2 M sodium formate (pH 3.6) followed by 10 cm in 4 M sodium formate (pH 3.6) at 25°C. Cellulose plates were developed by ascending chromatography in a solvent prepared by mixing 300 ml of 1 M ammonium acetate, pH 7.5, with 700 ml of 95% ethanol.

RESULTS

Synthesis of (γ -AmNS)NTPs—The structure of the ATP analog containing the fluorophore, 1-aminonaphthalene-5-sulfonate, attached via a γ -phosphoamidate bond is shown in Fig. 1. This fluorescent nucleotide, adenine-5'-triphosphoryl-1-(5-sulfonic acid)naphthylamide, has been termed (γ -AmNS)ATP (18). The synthesis and purification is simple and straight forward. Following a one-step reaction with a water-soluble carbodiimide, the reaction products are chromatographed on a DEAE-cellulose column. The fluorescent analog elutes after the unreacted nucleotide. Yields are good; they normally range from about 40 to 60% conversion of the NTP to the fluorescent derivative. The reaction proceeds well with all 4 NTPs. In addition, it is possible to synthesize the corresponding deoxy-NTPs. The analogs are quite stable at neutral pH and can be stored for several months at -20°C without significant degradation.

Effects of Venom Phosphodiesterase Digestion on the Spectroscopic Properties of the (γ -AmNS)NTPs—The absorption spectrum of (γ -AmNS)ATP is shown in Fig. 2. There is a broad band centered at 315 nm associated with the naphthalene ring ($\epsilon = 5580 \pm 150 \text{ M}^{-1} \text{ cm}^{-1}$), a distinct shoulder at 260

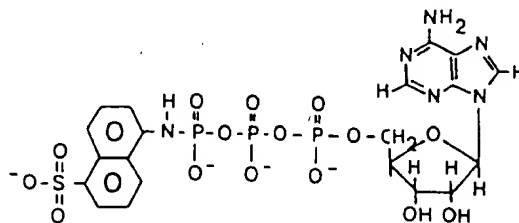


FIG. 1. Structure of (γ -AmNS)ATP.

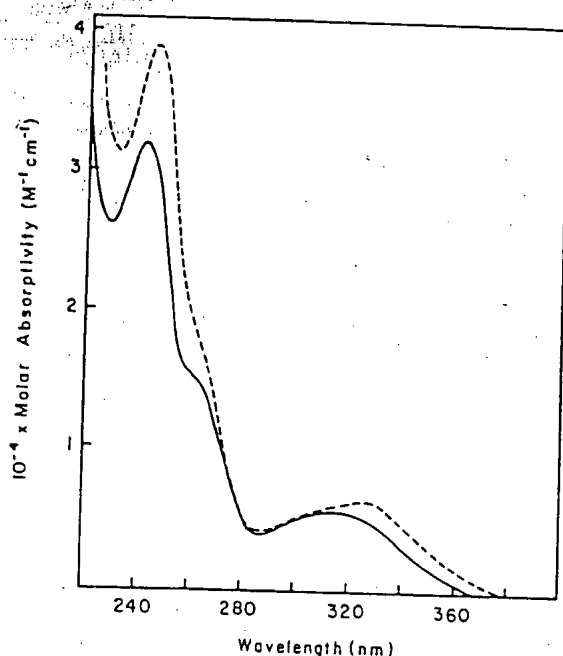


FIG. 2. Absorption spectrum of $(\gamma\text{-AmNS})\text{ATP}$. (—), before digestion with venom phosphodiesterase; (---), after digestion. Digestion was shown to produce AMP and AmNS-PP, by thin layer chromatography.

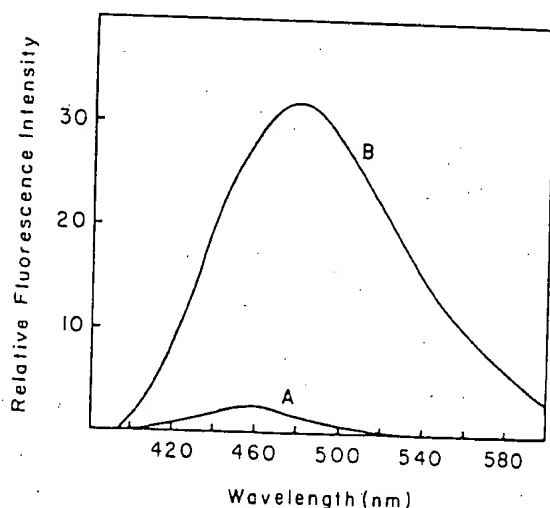


FIG. 3. Corrected fluorescence emission spectrum of $(\gamma\text{-AmNS})\text{UTP}$. Curve A, before digestion with venom phosphodiesterase; Curve B, after digestion. The excitation wavelength was 360 nm with a 10 nm excitation and emission band width.

nm due to the adenine ring, and a maximum at 242 nm ($\epsilon = 3.13 \pm 0.1 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$). Cleavage of the $\alpha\text{-}\beta$ -phosphoryl bond with venom phosphodiesterase produces a shift of the 315 nm band to 325 nm as well as a hyperchromicity of about 16%; the absorption at 260 nm increases by about 30%, and the absorption maximum at 242 nm shifts to 246 nm. The pyrophosphate derivative of AmNS, $(\text{AmNS})\text{PP}_i$, shows an absorption band at 325 nm ($\epsilon = 6500 \pm 100 \text{ M}^{-1} \text{ cm}^{-1}$), identical to that produced by digestion of $(\gamma\text{-AmNS})\text{ATP}$ with venom phosphodiesterase. This is about a 5 nm blue shift of the band found for AmNS which has a maximum at 330 nm.

The other $(\gamma\text{-AmNS})\text{NTPs}$ show very similar long wavelength absorption bands which also undergo large (8 to 10 nm) red shifts when the $\alpha\text{-}\beta$ -phosphoryl bonds are cleaved. There are also significant increases in the absorbance at 260 nm on digestion with venom phosphodiesterase. For example, $(\gamma\text{-AmNS})\text{UTP}$ shows about a 42% increase in the absorbance at 260 nm after digestion.

We have previously found that $(\gamma\text{-AmNS})\text{ATP}$ has a fluorescence emission maximum at 460 nm which shifts to 475 nm when the $\alpha\text{-}\beta$ -phosphoryl bond is cleaved (18). If excitation is performed at the isosbestic point ($\sim 315 \text{ nm}$), the fluorescence intensity at 500 nm increases by about 10% as a result of this cleavage. In contrast, the fluorescence intensity of $(\gamma\text{-AmNS})\text{UTP}$ increases manyfold when the $\alpha\text{-}\beta$ -phosphoryl bond is cleaved. If excitation is performed at 360 nm to take advantage of the shift in the absorption spectra, the fluorescence intensity at 500 nm increases about 14-fold when the analog is digested with venom phosphodiesterase (Fig. 3). Thus the fluorescence of $(\gamma\text{-AmNS})\text{UTP}$ is severely quenched. The quantum yield is only 0.086 as compared with 0.63 for $(\gamma\text{-AmNS})\text{ATP}$. Cleavage of the $\alpha\text{-}\beta$ phosphoryl bond of $(\gamma\text{-AmNS})\text{CTP}$ also produces an increase in fluorescence of about 8-fold, and a similar increase is observed with the deoxy-CTP analog. $(\gamma\text{-AmNS})\text{GTP}$ exhibits fluorescence properties similar to those found for $(\gamma\text{-AmNS})\text{ATP}$. Thus only the pyrimidine analogs show strongly quenched fluorescence.

Use of $(\gamma\text{-AmNS})\text{NTPs}$ for RNA Synthesis by DNA-dependent RNA Polymerase—The ATP and UTP analogs were examined for their ability to support template-dependent RNA synthesis with *E. coli* RNA polymerase. Table I shows that both are good substrates. The incorporation ranges from 60 to 80% of that found with the unmodified NTPs. Anthony *et al.* (26) and Downey and So (27) have shown that if the concentration of one NTP is varied and the others held constant at a relatively high concentration (0.4 mM), linear double reciprocal plots of velocity *versus* substrate can be obtained. Using this procedure, we have obtained apparent K_m and V_{max} values for $(\gamma\text{-AmNS})\text{ATP}$ and $(\gamma\text{-AmNS})\text{UTP}$ (Table II). For both, the maximum velocity is about 60 to 70% of that found for the normal substrates. The apparent K_m for the ATP analog is about the same as found for ATP; the value for the UTP analog is about double that found for UTP. Thus, the presence of the bulky naphthalene group does not significantly affect the ability of these analogs to serve as substrates for *E. coli* RNA polymerase. In contrast, they are poor substrates for wheat germ RNA polymerase II. The V_{max} for RNA polymerase II with $(\gamma\text{-AmNS})\text{ATP}$ or $(\gamma\text{-AmNS})\text{UTP}$ as a substrate is only about 15 to 20% of that found for the unmodified NTPs. In addition the K_m values are increased by about an order of magnitude (data not shown).

Initiation of RNA Chains with $(\gamma\text{-AmNS})\text{ATP}$ —Since purine NTPs are normally used to initiate RNA chains, the observation that $(\gamma\text{-AmNS})\text{ATP}$ supported efficient RNA synthesis with poly[d(A-T)]-poly[d(A-T)] as template suggested that the analog is used to initiate RNA chains. To demonstrate this directly, RNA was synthesized with $(\gamma\text{-AmNS})\text{ATP}$.

TABLE I
RNA synthesis by *Escherichia coli* RNA polymerase using $(\gamma\text{-AmNS})\text{ATP}$ or $(\gamma\text{-AmNS})\text{UTP}$

Reactions (0.1 ml) contained: 0.05 M Tris, pH 8, 0.05 M KCl, 0.01 M MgCl_2 , 10^{-3} M dithiothreitol, 4×10^{-4} M NTPs, 30 nmol of T7 DNA or poly[d(A-T)]-poly[d(A-T)], and 2 μg of holoenzyme. Samples were incubated 10 min at 37°C and precipitated with trichloroacetic acid.

Template	Nucleotide analog	$[\text{H}]\text{NMP}$ incorporated nmol	% Control
T7 DNA	None	1.89	100
	$(\gamma\text{-AmNS})\text{ATP}$	1.28	68
	$(\gamma\text{-AmNS})\text{UTP}$	1.54	81
poly[d(A-T)]-poly[d(A-T)]	None	1.37	100
	$(\gamma\text{-AmNS})\text{ATP}$	0.96	70

AmNS)ATP and [^3H]UTP as substrates and poly[d(A-T)]·poly[d(A-T)] as template. SDS was added to 0.1% and the reaction products were chromatographed on a Sephadex G-50 column equilibrated with 0.1% SDS. The fractions obtained were assayed for fluorescence and ^3H . Fig. 4 shows that there is a fluorescent peak which co-elutes with ^3H in the void volume. No ^3H or fluorescence eluted in the void volume if the synthesis was performed in the presence of rifampicin or if the reaction products were digested with RNase. Thus we conclude that (γ -AmNS)ATP is used to initiate RNA chains.

Unprimed Synthesis with (γ -AmNS)NTPs—*E. coli* RNA polymerase is known to catalyze the formation of poly(rA-rU)

TABLE II

Kinetic constants for (γ -AmNS)NTPs with *Escherichia coli* RNA polymerase

Reactions (0.1 ml) contained 1 to 2 μg of enzyme, 0.05 M Tris, pH 8, 0.05 M KCl, 10 mM MgCl₂, 1 mM dithiothreitol, 0.4 mM [^3H]GTP, [^3H]ATP, or [^3H]UTP, 8000 to 10,000 cpm/nmol, unlabeled NTPs 0.4 mM where necessary, 0.005 to 0.4 mM of the varying nucleotide, and 20 nmol of poly[d(A-T)]·poly[d(A-T)], or native calf thymus DNA were incubated for 5 min at 37°C, precipitated, filtered, and counted as described previously. V_{max} is expressed in nanomoles of [^3H]NMP incorporated/mg of enzyme /min.

Variable nucleotide	Template			
	Calf thymus DNA		Poly[d(A-T)]·poly[d(A-T)]	
	K_m μM	V_{max}	K_m μM	V_{max}
ATP	38	61		
(γ -AmNS)ATP	45	38		
UTP	31	59	53	72
(γ -AmNS)UTP	97	36	82	42

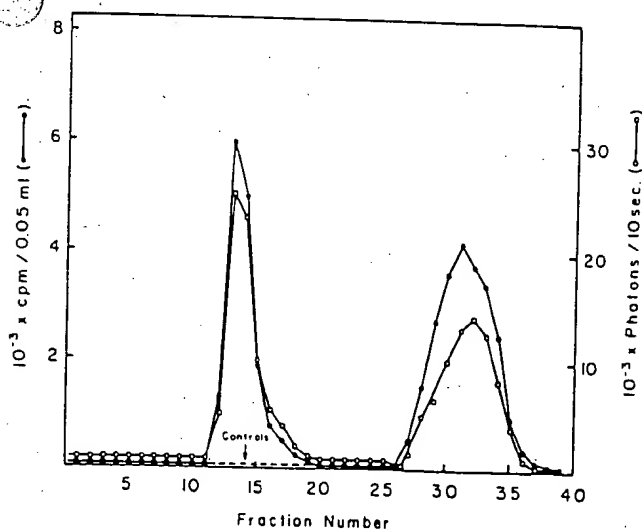


FIG. 4. RNA chain initiation with (γ -AmNS)ATP. RNA was synthesized in a 1 ml reaction containing 0.05 M Tris, pH 8, 0.05 M KCl, 2 mM dithiothreitol, 0.2 mM (γ -AmNS)ATP, 0.2 mM [^3H]UTP (5690 cpm/nmol), 150 nmol of poly[d(A-T)]·poly[d(A-T)], and 80 μg of *Escherichia coli* holoenzyme. After a 30-min incubation at 37°C, a 0.05-ml aliquot was precipitated with trichloroacetic acid and counted which revealed that a total 44 nmol of [^3H]UMP had been incorporated. The remaining sample was made 0.1% in SDS and chromatographed on a Sephadex G-25 column equilibrated with 0.01 M Na₂HPO₄, pH 7, containing 0.1% SDS. Fractions were assayed for ^3H (●—●) by liquid scintillation counting and for fluorescence (○—○) using an SLM single photon counting fluorescence spectrometer. Excitation was at 320 nm and emission was measured at 460 nm. The buffer background fluorescence, which corresponds to ~2000 photons/10 s has been subtracted, and the fluorescence of the included peak is reduced by a factor of 1000. Assuming that there is no change in the quantum yield of (γ -AmNS)ATP when incorporated into RNA, about 100 pmol of RNA chains were produced.

TABLE III

Template independent poly(rA-rU) synthesis with (γ -AmNS)NTPs by *Escherichia coli* RNA polymerase

Reactions (0.1 ml) contained: 0.05 M Tris, pH 8, 2 mM MnCl₂, 1 mM dithiothreitol, and 1 mM of the indicated NTPs. Samples were incubated for 2 h at 37°C, precipitated with trichloroacetic acid, and the precipitate collected on glass fiber filters. The filters, containing the precipitated product, were counted by liquid scintillation.

Nucleotides	[^3H]NMP incorporated nmol	% Control
ATP, [^3H]UTP	25.4	100
(γ -AmNS)ATP, [^3H]UTP	24.8	98
ATP, (γ -AmNS)[^3H]UTP	21.1	83
(γ -AmNS)ATP, (γ -AmNS)[^3H]UTP	7.5	30

in a template-independent reaction in the presence of Mn²⁺ and a high concentration of NTPs (28). Table III shows that both (γ -AmNS)ATP and (γ -AmNS)UTP can be used in this reaction. Moreover, the reaction will also proceed with only the analogs as substrate, although less efficiently.

DISCUSSION

The (γ -AmNS)NTPs should be excellent probes for the study of DNA-dependent RNA polymerases and perhaps other systems involving the utilization of nucleoside triphosphates. They have an absorption band located in the region 300 to 360 nm, well resolved from protein and nucleic acid absorption bands. This permits their selective excitation and thereby alleviates possible inner filter effects due to absorption by proteins or nucleic acids. Since the absorption band of the AmNS moiety overlaps the fluorescence emission spectra of proteins, these analogs can act as acceptors for resonance energy transfer from intrinsic protein fluorophores. Thus binding of (γ -AmNS)NTPs to proteins may produce quenching of protein fluorescence. If so, this property can be used to study both the equilibrium and kinetic aspects of protein-nucleotide interactions.

Although both the purine and pyrimidine analogs show essentially identical fluorescence emission spectra in terms of shape and emission maxima, only the pyrimidine analogs have strongly quenched fluorescence. This quenching involves interaction between the uridine and the naphthalene ring as evidenced by CD and NMR measurements.³ Studies of the fluorescence excited state lifetimes (data not shown) indicate that the quenching is of the dynamic type due to collisional interactions between the two rings.

It is not clear why only the pyrimidine analogs show strongly quenched fluorescence. Perhaps this reflects the fact that pyrimidines may assume a conformation which is especially favorable for stacking interactions between the two rings. For pyrimidines, the glycosidic bond angle χ normally ranges from 25 to 105° while for purines it ranges from 3 to 55° (29). Inspection of space filling models of (γ -AmNS)NTPs suggest that stacking would be most favored for χ values around 90 to 100°, i.e. when the rings are co-planar. Alternatively, since the purines are larger than the pyrimidines, it is possible that the sulfonic acid moiety prevents close interaction of the purine and naphthalene rings.

The finding of intramolecular quenching interactions between the pyrimidine and naphthalene rings is not entirely surprising since similar observations have been made for several other related systems. Spencer (30) found that the fluorescence of NADH is strongly quenched by collisional interaction between the adenine and nicotinamide rings. Bar-

³ L. R. Yarbrough and J. Hock, unpublished data.

rio *et al.* (31) found that the fluorescence of the etheno derivative of FAD is quenched by both dynamic and static mechanisms. More recently, Leonard *et al.* (11) determined that the symmetrical anhydride of lin-benzo AMP which was linked through the 5'-phosphorus residues is almost nonfluorescent due to ring-ring interactions. Thus stacking which results in fluorescence quenching is a common occurrence.

Our observations that the (γ -AmNS)NTPs are good substrates for *E. coli* RNA polymerase and that the ATP analog can be used to initiate RNA chains are in good agreement with the results obtained by Grachev and Zaychikov (20) for the related analog, ATP- γ -anilidate. They found that this analog was incorporated with 50 to 60% of the efficiency of ATP and that it could apparently be used to initiate RNA chains. Armstrong and Eckstein (32) have also synthesized analogs containing either a fluorine atom, a methyl group, or a phenyl group attached to the γ -phosphate of ATP. They found that these analogs were incorporated only about 10 to 15% as efficiently as ATP. Inhibition studies showed that the inhibition constants for these analogs were on the order of 1 to 2 mM indicating that they bind about an order of magnitude more weakly than the normal nucleotide. They suggested that this was due to the loss of a negative charge on the γ -phosphate. However, our data, as well as the data of Grachev and Zaychikov, suggest that the loss of a negative charge does not greatly alter the ability of a nucleotide to bind to the enzyme or to be incorporated efficiently. Perhaps a more likely explanation is that modification may alter nucleotide conformation and/or interaction with divalent cations. For example, we have found that an analog containing AmNS attached via a 5-atom bridge is a very poor substrate for either *E. coli* or wheat germ RNA polymerase.⁴

The ability of these analogs to serve as substrates for RNA polymerase may also reflect steric properties of the nucleotide binding site. The bulky naphthalene group might prevent the nucleotide from assuming the proper orientation when it binds to the enzyme. This could explain the significant decrease in V_{max} found for wheat germ RNA polymerase with the analogs as substrates. These analogs could thus complement the "dimensional probes" synthesized by Leonard and co-workers, the lin-benzo ATP analogs (11).

In summary, we have shown that the (γ -AmNS)NTPs are excellent substrates for *E. coli* DNA-dependent RNA polymerase. This, along with their desirable spectroscopic properties, makes them excellent tools for study of the kinetics and mechanism of RNA synthesis (33).

Acknowledgement—We thank Dr. R. Hirschberg for a critical reading of the manuscript.

⁴ L. R. Yarbrough and T. L. Carro, unpublished data.

REFERENCES

1. Yount, R. G., Babcock, D., Ballantyne, W., and Ojala, D. (1971) *Biochemistry* 10, 2484-2489
2. Yount, R. G. (1975) *Adv. Enzymol.* 43, 1-56
3. Goody, R. S., and Eckstein, F. (1971) *J. Am. Chem. Soc.* 93, 6252-6257
4. Hampton, A., Perini, F., and Harper, P. J. (1973) *Biochemistry* 12, 1730-1736
5. Tonomura, Y., Imamura, K., Ikehara, M., Uno, H., and Harada, F. (1967) *J. Biochem. (Tokyo)* 61, 460-472
6. Secrist, J. A., Barrio, J. R., and Leonard, N. J. (1972) *Science* 175, 656-647
7. Darlix, J. L., Fromageot, P., and Reich, E. (1971) *Biochemistry* 10, 1525-1531
8. Ward, D. C., Reich, E., and Stryer, L. (1969) *J. Biol. Chem.* 244, 1228-1237
9. McClure, W. R., and Scheit, K.-H. (1973) *FEBS Lett.* 32, 267-269
10. Scopes, D. I. C., Barrio, J. R., and Leonard, N. J. (1977) *Science* 195, 296-298
11. Leonard, N. J., Scopes, D. I. C., VanDerLijn, P., and Barrio, J. R. (1978) *Biochemistry* 17, 3677-3685
12. Wiegand, G., and Kaleja, R. (1976) *Eur. J. Biochem.* 65, 473-479
13. Chamberlin, M. J. (1974) *Ann. Rev. Biochem.* 43, 721-775
14. Auld, D. S., Latt, S. A., and Vallee, B. L. (1972) *Biochemistry* 11, 4994-4999
15. Latt, S. A., Auld, D. S., and Vallee, B. L. (1970) *Proc. Natl. Acad. Sci. U. S. A.* 67, 1383-1389
16. Latt, S. A., Auld, D. S., and Vallee, B. L. (1972) *Biochemistry* 11, 3015-3022
17. Wu, C. W., and Wu, F. Y. H. (1973) *Fed. Proc.* 32, 645
18. Yarbrough, L. R. (1978) *Biochem. Biophys. Res. Commun.* 81, 35-41
19. Kumar, S. A., Krakow, J. S., and Ward, D. C. (1977) *Biochim. Biophys. Acta* 477, 112-124
20. Grachev, M. A., and Zaychikov, E. F. (1974) *FEBS Lett.* 49, 163-166
21. Babkina, G. T., Zarytova, V. F., and Knorre, D. G. (1975) *Biorg. Khim.* 1, 611-615
22. Burgess, R. R., and Jendrisak, J. J. (1975) *Biochemistry* 14, 4634-4638
23. Jendrisak, J. J., and Burgess, R. R. (1975) *Biochemistry* 14, 4639-4645
24. Melhuish, W. H. (1961) *J. Phys. Chem.* 65, 229-235
25. Scott, T. G., Spencer, R. D., Leonard, N. J., and Weber, G. (1970) *J. Am. Chem. Soc.* 92, 687-695
26. Anthony, D. D., Wu, C. W., and Goldthwait, D. A. (1969) *Biochemistry* 8, 246-256
27. Downey, K. M., and So, A. G. (1970) *Biochemistry* 9, 2520-2525
28. Krakow, J. S. (1968) *Biochim. Biophys. Acta* 166, 459-465
29. Bloomfield, V. A., Crothers, D. M., and Tinoco, I. (1974) *Physical Chemistry of Nucleic Acids*, pp. 1-66, Harper & Rowe, New York
30. Spencer, R. (1970) *Fluorescence Lifetimes: Theory, Instrumentation and Application of Nanosecond Fluorimetry*, pp. 221-253, Ph.D. thesis, University of Illinois
31. Barrio, J. R., Tolman, G. L., Leonard, N. J., Spencer, R. D., and Weber, G. (1973) *Proc. Natl. Acad. Sci. U. S. A.* 70, 941-943
32. Armstrong, V. W., and Eckstein, F. (1976) *Eur. J. Biochem.* 70, 33-38
33. Schlageck, J., Baughman, M., and Yarbrough, L. R. (1979) *J. Biol. Chem.* 254, 12074-12077

11-621.
5-2064.
(1974) Anal. Biochem.

055.
Biophys. Acta, 479.

Strud, R. & Prydz.

unochem. 1, 321.

immunol. 2, Suppl.

immunol. Methods, 13.

15, 450-456.
(1976) J. Immunol.

973) Scand. J. Immunol.

Lab. Clin. Med. 83.

Scand. J. Immunol.

nd. J. Immunol. 6.

nochem. 3, 155.

Fluorescent Derivatives of Yeast tRNA^{Phe}

Wolfgang WINTERMEYER and Hans G. ZACHAU

Institut für Physiologische Chemie, Physikalische Biochemie und Zellbiologie der Universität München

(Received December 13, 1978/March 16, 1979)

The preparation of four fluorescent derivatives of tRNA^{Phe} (yeast) and their characterization by chemical, spectroscopic, and biochemical methods is described. The derivatives are prepared by replacing wybutine (position 37 in the anticodon loop) or NaBH₄-reduced dihydrouracil (positions 16/17 in the hU loop) with ethidium or proflavine; they are isolated by reversed-phase chromatography (RPC-5). All tRNA^{Phe}-dye derivatives are aminoacylated by yeast phenylalanyl-tRNA synthetase to at least 80% of the charging capacity of the unmodified tRNA^{Phe} with an unchanged *K_m* (0.2 μM) and a *V* lowered by 30-50%. They exhibit good to excellent activity in the aminoacylation assay with synthetase from *Escherichia coli*. It is concluded that the insertion of the dyes does not seriously disturb essential elements of the native tRNA^{Phe} structure.

The dyes are bound via *N*-ribosylic linkages. The appearance of isomeric tRNA^{Phe}-ethidium derivatives is attributed to the involvement of the different amino groups of ethidium in the condensation. In addition, there are indications for the existence of α and β anomers of the tRNA-dye compounds.

The dyes are rigidly fixed to their position in the tRNA molecule by stacking interactions with the neighboring bases.

The ethidium probes show Mg²⁺-induced changes of the tRNA conformation which are paralleled by changes of the rate of aminoacylation. On the basis of this observation it is hypothesized that conformational flexibility of the tRNA molecule is a functionally important feature of the tRNA structure.

Fluorescent derivatives of tRNA have proven to be useful for studies on the structure of tRNA and its various functions. The majority of the fluorescent probes were introduced into tRNA by covalent attachment to the periodate-oxidized 3'-end [1] or to odd bases carrying unique functional groups [2-4].

Dedicated to Professor Fritz Lipmann on the occasion of his 10th birthday.

Abbreviations. YWye, wybutine (rare base at position 37 of yeast tRNA^{Phe}, formerly called Y⁺ or Y base); yW, the nucleoside wyosine; yW(red) and hU(red), NaBH₄-reduced wyosine and dihydrouridine (ureidopropanol riboside), respectively; EtdBr, ethidium bromide (2,7-diamino-9-phenyl-10-ethyl-phenanthridinium bromide); Prf, proflavine; tRNA^{Phe}_{Wy}, tRNA^{Phe} lacking wybutine; tRNA^{Phe}_{Etd37}, tRNA^{Phe} carrying ethidium in place of wybutine; tRNA^{Phe}_{Etd37B} and tRNA^{Phe}_{Etd37C}, separated species of tRNA^{Phe}_{Etd37} in which ethidium is probably bound via the 7-amino and the 2-amino groups respectively (see text); tRNA^{Phe}_{Etd16/17}, tRNA^{Phe} in which ethidium replaces dihydrouracil in position 16 or 17 (see text); E(B) and E(C) the B and C forms of the ribosyl derivatives of ethidium (see text); P, the ribosyl derivative of proflavine.

Enzymes. Phenylalanyl-tRNA synthetase (EC 6.1.1.20); T₁ RNAase (EC 3.1.4.8); pancreatic RNAase (EC 3.1.4.22); phosphodiesterase from snake venom (EC 3.1.4.1) and spleen (EC 3.1.4.18); alkaline phosphatase (EC 3.1.3.1).

Since chemical modification of the 3'-end inactivates the tRNA and addition of bulky groups in many cases impairs its functions, we have developed an alternative procedure by which odd bases are replaced with fluorescent dyes [5,6]. The replacement involves a two-step procedure: a ribosylic aldehyde group is created in the tRNA by selective excision of a base which subsequently can be condensed with a fluorophor possessing either a primary amino or a hydrazino group. The procedure has been used for the insertion of the aromatic amines proflavine and ethidium in the place of wybutine or dihydrouracil in tRNA^{Phe} and in the dihydrouracil positions in tRNA^{Ser} from yeast [5,6] as well as for the insertion of hydrazine derivatives at the wybutine position [7]. The proflavine and ethidium derivatives to tRNA^{Phe} have been successfully applied in studies on synthetase interactions [8], tRNA conformation, and ribosome interaction [9-11] (summary [12]). However, the preparation, structural characterization, and spectroscopic properties of several of the tRNA^{Phe}-dye derivatives used have not yet been described in full. This is accomplished in the present paper.

MATERIALS AND METHODS

tRNA

tRNA^{Phe} was isolated from brewer's yeast tRNA (Boehringer Mannheim) as described previously [13] and accepted 1.5–1.7 nmol Phe/*A*₂₆₀ unit. tRNA^{Phe}_{Wye} was prepared from tRNA^{Phe} [14] and accepted around 1.3 nmol Phe/*A*₂₆₀ unit. Both tRNA^{Phe} and tRNA^{Phe}_{Wye} moved as single bands on polyacrylamide gel electrophoresis in the presence of urea [15].

Enzymes

Phenylalanyl-tRNA synthetase from yeast (3.5 U/mg [16]) was donated by U. Pachmann who prepared it by a published procedure [16] including an affinity elution step [17]. Phenylalanyl-tRNA synthetase from *E. coli* K10 (45 nmol Phe mg⁻¹ h⁻¹) was a gift of A. Böck [18]. T₁ and T₂ RNAase, were purchased from Sankyo, pancreatic RNAase, spleen and snake venom phosphodiesterase, and alkaline phosphatase from *E. coli* were from Boehringer, Mannheim. The activity specifications of the manufacturers were used.

Chemicals and Materials for Chromatography

Ethidium bromide (Serva, Heidelberg) was homogeneous on paper electrophoresis and thin-layer chromatography in three solvent systems and was used as purchased; proflavine (Fluka, Buchs) was purified by repeated crystallizations of the free base from ethanol/water. Phenol and ether were distilled shortly before use. L-[¹⁴C]Phenylalanine (specific activities 10 and 59 Ci/mol) was purchased from The Radiochemical Center (Amersham), NaB[³H₄] (200–400 Ci/mol) from New England Nuclear. NaBH₄ (for synthetic purposes) and all other chemicals (analytical grade) were from Merck (Darmstadt). Column chromatography was performed on DEAE-cellulose (DE-52, Whatman), DEAE-Sephadex A-25 (Pharmacia), and benzoylated DEAE-cellulose (Boehringer, Mannheim). The materials for reversed-phase chromatography (RPC-5), polychlorotrifluoroethylene and trioctylmethylammonium bromide, had been purchased from Serva (Heidelberg).

Reductions with NaBH₄

In the standard procedure to a solution of 50 *A*₂₆₀ units tRNA^{Phe}/ml 0.2 M Tris-HCl, pH 7.5, in ice 0.1 vol. of a solution of 100 mg NaBH₄/ml 0.01 M KOH was added. After 30 min the reaction was terminated by adjusting the pH to 4–5 with 6 M acetic acid. The reduced tRNA was isolated and washed by three ethanol precipitations.

Reductions with NaB[³H₄] were carried out at pH 9.8 [19] in order to slow down the hydrolysis of the reagent.

Preparation of tRNA^{Phe}-Dye Compounds

The previously published procedures [5,6] were used for the incorporation of proflavine and ethidium respectively, both at positions 37 or 16/17 of tRNA^{Phe}.

Aminoacylation Assay

If not stated otherwise the homologous aminoacylation assay with phenylalanyl-tRNA synthetase from yeast was performed as previously described [14]. The reaction mixture (0.1 ml) contained 25 mM Tris-HCl, pH 7.5, 20 mM MgCl₂, 10 mM ATP, 50 μM L-[¹⁴C]phenylalanine (10 Ci/mol), 0.05–0.07 *A*₂₆₀ unit tRNA^{Phe} (which had been preincubated in 1 mM Tris-HCl, pH 7.5, 1 mM MgCl₂ for 15 min at 37°C), and 0.1–0.2 mU phenylalanyl-tRNA synthetase. The mixture was incubated at 37°C for 15 min. Michaelis-Menten kinetics were measured in 0.4-ml incubation mixtures of the same composition except that L-[¹⁴C]phenylalanine with higher specific radioactivity (59 Ci/mol) and 0.15 mU phenylalanyl-tRNA synthetase were used; the tRNA concentration was varied between 0.03 μM and 0.47 μM (1 *A*₂₆₀ unit was taken to represent 1.75 nmol). Incubation was for 1 min at 21°C.

Nuclease Digestions

tRNA^{Phe}-dye compounds (20–50 *A*₂₆₀ units/ml) were digested with various nucleases in 10 mM Tris-HCl, pH 7.5, for 3 h at 25°C. The concentrations of nucleases were (U/ml): T₁ RNAase (250), pancreatic RNAase (3), T₂ RNAase (20). For chromatographic separations of the digestion products the digestion mixtures were made 7 M in urea by addition of 10 M urea and applied to DEAE-cellulose columns.

Chromatographic Procedures

Reversed-phase Chromatography (RPC-5). The adsorbent was prepared for use following procedure C of Pearson et al. [20]. Columns (0.3 × 60 cm for up to 100 *A*₂₆₀ units tRNA) were run at 24°C at approximately 40 bar (4 MPa) pressure (Labotron, HKP 50, Kontron) in 10 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 0.4 M NaCl and developed with 800-ml gradients from 0.4 M to 0.8 M NaCl in the same buffer. The tRNA was isolated from the pooled fractions by ethanol precipitation, followed by two additional precipitations. This procedure led to tRNA preparations with good amino acid acceptance. Dialysis and concentration by flash evaporation prior to

W. Winterme
the precip
led to tR
DEAE
of tRNA
were chro
(0.3 × 70 c
0.01 M an
7 M urea;
at a flow
chromato
were pool
to DEAE
with 0.01
washing v
with a lin
0.5 M am
The se
tography
ammoniu
High-volt.
Schüll, 20
monium e
Spectroph
Absorpti
absorbanc
meter. Fl
Elmer M1
fluorimete
9820A cal
spectra we
of the det
calculated
source, as
thermopil
the spectr
excitation
chromato
RESULT
Chemical
Chrom
specific i
at the po
been rep
separated
DEAE-ce
elution p
of tRNA
at positic
and exhibit
Reversed-
sulted in
tRNA^{Phe}
of tRNA

ried out at
olysis of the

the precipitation were avoided, since they sometimes
led to tRNA fractions with low charging capacity.

DEAE-cellulose Chromatography. Nuclease digests of tRNA^{Phe}-dye compounds (up to 40 A_{260} units) were chromatographed on DEAE-cellulose columns (0.3 × 70 cm) with a linear gradient of 250 ml each of 0.01 M and 0.3 M NaCl in 10 mM Tris-HCl, pH 7.5, 7 M urea; the columns were run at 24°C and 15–20 bar at a flow rate of approximately 0.5 ml/min. For rechromatography oligonucleotide-containing fractions were pooled, diluted fivefold with water and applied to DEAE-cellulose columns (0.5 × 5 cm) equilibrated with 0.01 M ammonium bicarbonate, pH 8; after washing with the same buffer elution was performed with a linear gradient of 100 ml each of 0.01 M and 0.5 M ammonium bicarbonate, pH 8.

The solvent system used for thin-layer chromatography on cellulose plates consisted of 4 vol. 1 M ammonium acetate, pH 7.5, and 1 vol. 2-propanol. High-voltage electrophoresis on paper (Schleicher and Schüll, 2043b) was carried out using a 0.05 M ammonium acetate buffer, pH 7.0.

Spectrophotometric Measurements

Absorbance was measured with a Zeiss PMQII, absorbance spectra with a Cary 118 spectrophotometer. Fluorescence was measured with a Perkin-Elmer MPF-2A and a Schoeffel RRS 1000 spectrofluorimeter which was interfaced to a Hewlett-Packard 9820A calculator/9862 plotter combination. Emission spectra were corrected for the wavelength dependence of the detection system. The correction factors were calculated from the energy distribution of the excitation source, as measured with a temperature-compensated thermopile (type CA1, Kipp and Zonen, Delft), and the spectrum measured on the emission side when the excitation light was reflected on the emission monochromator with a magnesium oxide screen [21].

RESULTS

Chemical Characterization of tRNA^{Phe}_{Eid37}

Chromatographic Separation of Two Isomers. The specific incorporation of ethidium into tRNA^{Phe}_{Wy} at the position vacated by excision of wybutine has been reported [5]. The product, tRNA^{Phe}_{Eid37}, could be separated from unreacted tRNA^{Phe}_{Wy} by benzoylated DEAE-cellulose chromatography. In addition the elution profile indicated the separation of two species of tRNA^{Phe}_{Eid37}, both of which contain a single ethidium at position 37 (see oligonucleotide analysis below) and exhibit different fluorescence quantum yields [5]. Reversed-phase chromatography (RPC-5; [20]) resulted in a much improved separation of unlabeled tRNA^{Phe}_{Wy} (first eluted peak) and of the two species of tRNA^{Phe}_{Eid37}, which according to the elution profile

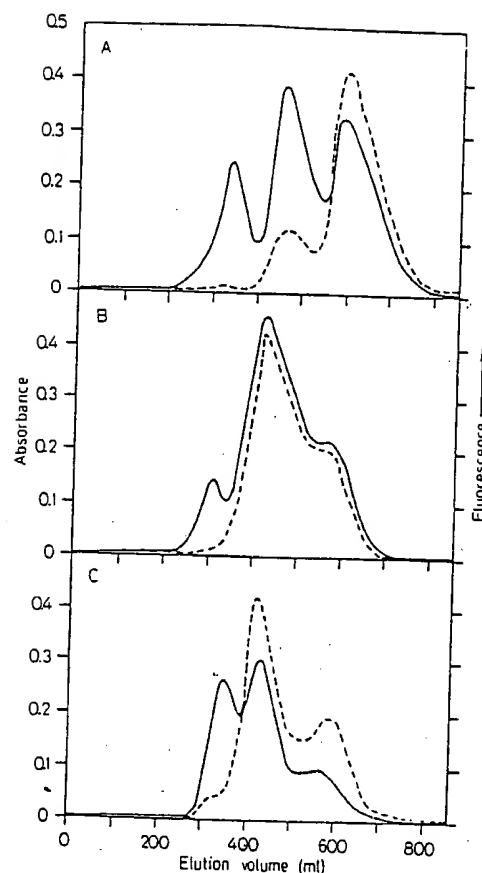


Fig. 1. Chromatography of tRNA^{Phe}_{Eid37} (A), tRNA^{Phe}_{Prf37} (B) and tRNA^{Phe}_{Eid16/17} (C) on reversed-phase (RPC-5) columns. Absorbance was measured at 260 nm (—), fluorescence (---) of ethidium at 590 nm (excitation at 470 nm), the one of proflavine at 500 nm (excitation at 460 nm).

are designated B and C (Fig. 1A). It should be mentioned that also from benzoylated DEAE-cellulose tRNA^{Phe}_{Wy} is eluted first but the order of elution of the two fluorescent species is reversed. The individual species retained their original elution position upon rechromatography on RPC-5. This observation makes it unlikely that the appearance of two species is due only to conformational differences; it rather suggests that species B and C are chemical isomers which do not interconvert under the conditions of chromatography and isolation (ethanol precipitation and phenol extraction). The formation of two isomeric condensation products of ethidium with ribose may be due to condensation at the two different amino groups of ethidium; pK values of 2.43 and 0.713 have been reported for the 7-amino and the 2-amino groups respectively [22]. In addition, N-ribosylic condensation products may be present as α and β anomers. However, the appearance of species B and C of tRNA^{Phe}_{Eid37} does not seem to reflect a chromatographic separation of α and β anomers because the same elution pattern was obtained after tRNA^{Phe}_{Eid37} reduction with NaBH₄ [5], which should eliminate a heterogeneity due to the presence of α and

gous amino-
A synthetase
described [14].
25 mM Tris-
ATP, 50 μ M
0.07 A_{260} unit
ted in 1 mM
min at 37°C),
nthesase. The
in. Michaelis-
ml incubation
t that L-[¹⁴C]-
activity (59 Ci/
A synthetase
was varied bet-
t was taken to
for 1 min at

A_{260} units/ml)
1 10 mM Tris-
(concentrations of
50), pancreatic
omatographic
the digestion
dition of 10 M
columns.

(RPC-5). The
ag procedure C
10 cm for up to
24°C at ap-
re (Labotron,
-HCl, pH 7.5;
veloped with
M NaCl in the
om the pooled
llowed by two
re led to tRNA
ccf ce. Di-
rat. prior to

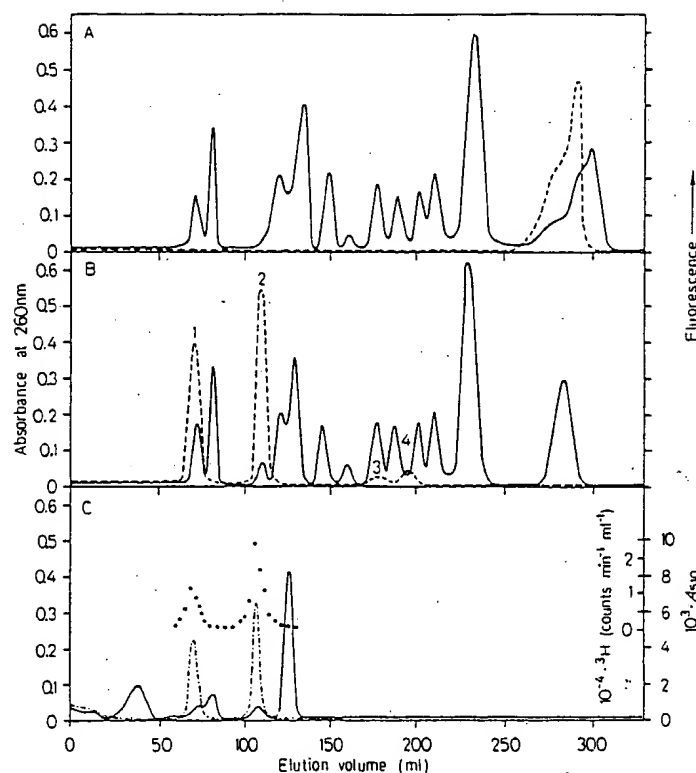


Fig. 2. Chromatography of T_1 RNAase digests of $tRNA_{Eid37}^{Phe}$ (A), $tRNA_{Eid37B}^{Phe}$ (B) and of the ethidium-containing dodecanucleotides from Fig. 7 (C) on DEAE-cellulose. A_{260} (—); A_{510} (---); ethidium fluorescence, measured as in Fig. 1 (---); 3H radioactivity (●)

β anomers. Positive evidence favoring the involvement of the two amino groups of ethidium in the condensation with $tRNA_{Ywe}^{Phe}$ has been obtained by comparing species B and C of $tRNA_{Eid37}^{Phe}$ with respect to the rates of ethidium excision at acidic pH. The hydrolysis could be followed by an increase (species B) or a decrease (species C) of ethidium fluorescence. The kinetic analysis revealed that ethidium is excised from $tRNA_{Eid37B}^{Phe}$ nearly four times faster ($t_{1/2} = 6$ min at $20^\circ C$ in 0.1 M acetate buffer, pH 4.0) than from $tRNA_{Eid37C}^{Phe}$ ($t_{1/2} = 21$ min). In view of the pK values mentioned above this result suggests that in species B and C of $tRNA_{Eid37}^{Phe}$ the ethidium is bound via its 7-amino and 2-amino groups, respectively.

Isomeric Ethidium-Containing Oligonucleotides Are Derived from Species B and C of $tRNA_{Eid37}^{Phe}$ by Nuclease Digestions. The chromatographic pattern of the T_1 RNAase digestion products of a mixture of $tRNA_{Ywe}^{Phe}$, $tRNA_{Eid37B}^{Phe}$, and $tRNA_{Eid37C}^{Phe}$ is shown in Fig. 2A. Ethidium fluorescence was observed in two oligonucleotides which were eluted just in front of the dodecanucleotide (—Ywe). As judged from the relative intensity of ethidium fluorescence, the first is derived from species B, the second one from species C. This result confirms that in both species of $tRNA_{Eid37}^{Phe}$ the dye is located only in the correct position in the anticodon loop [5]. The slightly different chromatographic (Fig. 2A) and electrophoretic

(Table 1) properties of the B and C forms of the ethidium-containing dodecanucleotide seem to be due to differences in shape. Depending on the steric arrangement the positively charged ring nitrogen of the dye may screen backbone charges to different extents and thereby cause the observed differences.

Complete Digestion Yields Anomeric Ethidium Ribosides. Species B and C of $tRNA_{Eid37}^{Phe}$ were digested separately with a mixture of nucleases (T_2 RNAase, snake venom and spleen phosphodiesterase, alkaline phosphatase). Thin-layer chromatography revealed that both species yield two fluorescent spots, i.e. two forms of the expected ethidium ribofuranosides (R_f values 0.12 and 0.34, same for species B and C; free Etd: 0.25). When extracted from the plate by phenol and rechromatographed each of the spots again yielded the same two spots indicating an isomerization of the ethidium ribofuranoside during isolation. Two ethidium-containing compounds were also observed when the products of T_2 RNAase digestion of both species B and C of $tRNA_{Eid37}^{Phe}$ were separated by paper electrophoresis (R_{AP} values 0.22 and 0.37; free Etd: —0.06). These observations are best explained by assuming that both species B and C yield upon total digestion a mixture of α and β anomers of the respective ethidium ribofuranosides. The anomers are apparently stable during chromatography, but interconvert during isolation.

Table 1. Electrophoretic mobilities of dye-containing oligonucleotides

The oligonucleotides were obtained from the indicated tRNA^{Phe} derivative by digestion with T₁ (T₁) or pancreatic (pan) RNAase. 18% polyacrylamide gels containing 7 M urea were run at pH 8.6 [15]. The bands were made visible by ultraviolet light (ethidium or proflavine-containing oligonucleotides) and by staining with Stains-all. The mobilities are given relative to the bromphenol blue marker (R_B value)

Region of tRNA	Oligonucleotide	tRNA	RNAase	R_B value
Anticodon loop	A-Cm-U-Gm-A-A-yW-A-ψ-m ⁵ C-U-Gp	tRNA ^{Phe}	T ₁	0.535
	A-Cm-U-Gm-A-A-Rib-A-ψ-m ⁵ C-U-Gp	tRNA ^{Phe} _{Ywy}	T ₁	0.580
	A-Cm-U-Gm-A-A-E(B)-A-ψ-m ⁵ C-U-Gp	tRNA ^{Phe} _{Eid37C}	T ₁	0.535
	A-Cm-U-Gm-A-A-E(C)-A-ψ-m ⁵ C-U-Gp	tRNA ^{Phe} _{Eid37B}	T ₁	0.510
	A-Cm-U-Gm-A-A-P-A-ψ-m ⁵ C-U-Gp	tRNA ^{Phe} _{Prf37}	T ₁	0.535
	Gm-A-A-yW-A-ψp	tRNA ^{Phe}	pan	0.730
	Gm-A-A-E(B)-A-ψp	tRNA ^{Phe} _{Eid37B}	pan	0.730
	Gm-A-A-E(C)-A-ψp	tRNA ^{Phe} _{Eid37C}	pan	0.700
	G-G-G-A-G-A-G-Cp	tRNA ^{Phe}	pan	0.690
	A-G-hU(red)-E(B)-G-G-G-A-G-A-G-Cp	tRNA ^{Phe} _{Eid16/17}	pan	0.595
hU loop	A-G-hU(red)-E(C)-G-G-G-A-G-A-G-Cp	tRNA ^{Phe} _{Eid16/17}	pan	0.525
	A-G-E(B)-E(B)-G-G-G-A-G-A-G-Cp	tRNA ^{Phe} _{Prf16/17}	pan	0.475
	A-G-hU(red)-P-G-G-G-A-G-A-G-Cp	tRNA ^{Phe} _{Prf16/17}	pan	0.540

The anomers of an ethidium ribofuranoside should yield the same compound upon reduction followed by reoxidation to restore the phenanthridinium system. However, total digestion experiments with NaBH₄-reduced and reoxidized tRNA^{Phe}_{Eid37B} and tRNA^{Phe}_{Eid37C} [5] analogous to those described above gave only inconclusive results. This may be due to side reactions during reduction and/or reoxidation which cannot be defined at the present time.

Characterization of tRNA^{Phe}_{Prf37}

The previously described separation of two species of tRNA^{Phe}_{Prf37} on benzoylated DEAE-cellulose [5] was improved by using reversed-phase chromatography (RPC-5) (Fig. 1B). According to the oligonucleotide analysis, both compounds contain one proflavine at position 37 [5]. The quantum yield of proflavine fluorescence is the same in the two compounds. After reduction with NaBH₄ the tRNA^{Phe}_{Prf37} was eluted from reversed-phase chromatography in a single peak, indicating that the heterogeneity of the unreduced compound is due to the presence of α and β anomers. It should be mentioned that in contrast to the ethidium-containing compounds, the proflavine-containing dodecanucleotides obtained by digesting the mixture of the two species of tRNA^{Phe}_{Prf37} with T₁ RNAase appear in one symmetric peak upon column chromatography under the conditions of Fig. 2 [5] and have the same mobility in disc gel electrophoresis (Table 1).

Characterization of tRNA^{Phe}-Dye Compounds Labeled in the hU Loop

Reduction of Dihydrouracil in tRNA^{Phe}. Dihydrouracil in tRNA has to be reduced in order to be sus-

ceptible to the replacement by amines. The NaBH₄ reduction of dihydrouracil had previously been performed at pH 9.8 [19]. However, as determined spectrophotometrically [23], a complete reduction of dihydrouracil can be achieved under much milder conditions (pH 7.5, 0°C) [24]. Besides dihydrouracil, 7-methylguanosine and wybutine are also affected. These side reactions cannot be avoided since under any condition dihydrouracil in tRNA^{Phe} is reduced by NaBH₄ at a slower rate than both 7-methylguanosine and wybutine. At least part of the reduced 7-methylguanosine is reoxidized to the starting compound by reaction with oxygen during isolation. It has been shown that the modification by reduction of wybutine [19] and 7-methylguanosine [13] has little or no effect on the activities of tRNA^{Phe} in biochemical assay systems (see also below).

Preparation and Isolation of tRNA^{Phe}_{Eid16/17} and tRNA^{Phe}_{Prf16/17}. Previously the replacement of reduced dihydrouracil by ethidium had been performed at pH 3 [6]. However, the replacement reaction proceeds equally well at pH 4.3 [24]; this condition is much to be preferred, since it avoids excision of wybutine (see below). According to absorbance measurements, 1.1 mol ethidium were incorporated/mol reduced tRNA^{Phe}, whereas non-reduced tRNA^{Phe} did not accept measurable amounts of dye. The incorporation of Prf was also found to be strictly dependent on the progress of dihydrouracil reduction, both in tRNA^{Phe} and tRNA^{Phe}_{Ywy} (Fig. 3). The incorporation of proflavine into fully reduced tRNA^{Phe} was complete after 2 h [24]. Reversed-phase chromatography (RPC-5) of the products of ethidium incorporation into reduced tRNA^{Phe} again separated two ethidium-containing species from non-labeled tRNA^{Phe} (Fig. 1C); only the major fluorescent species was used for further experiments and is designated

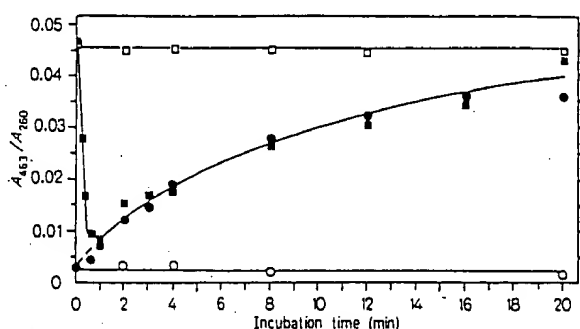


Fig. 3. Incorporation of proflavine into reduced tRNA. Per point 2 A_{260} units of $tRNA^{Phe}$ (●, ○) or $tRNA^{Phe_{wy}}$ (■, □) were treated with $NaBH_4$ (standard procedure in Materials and Methods) for various times. The controls were incubated under the same conditions without addition of $NaBH_4$. The isolated tRNA samples were reacted with proflavine as previously described [5]. The absorbance at 463 nm and 260 nm was measured for the $NaBH_4$ -treated (●, ■) and the control samples (○, □)

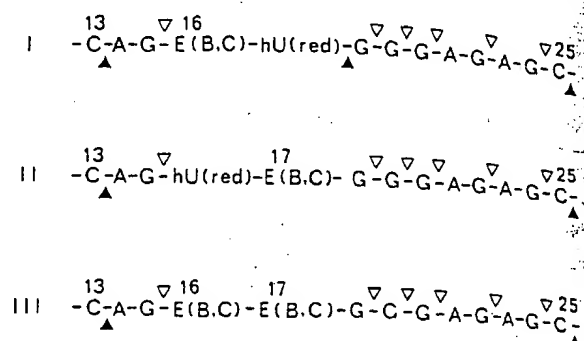


Fig. 4. Cleavages in the hU region of $tRNA^{Phe}_{Eid16}$ (I), $tRNA^{Phe}_{Eid17}$ (II) and $tRNA^{Phe}_{Eid16/17}$ (III) with T_1 (▽) and pancreatic (▲) RNAase. In I and II the ethidium riboside is present in both B and C forms giving rise to chromatographically different ethidium-containing oligonucleotides (see text). In III the distribution of B and C forms has not been determined. In II no cleavage was observed between hu(red) and E (B, C) (see text)

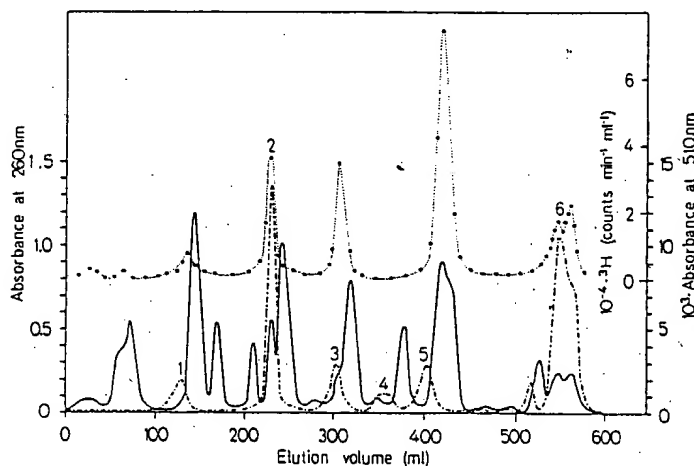


Fig. 5. DEAE-cellulose chromatography of a pancreatic RNAase digest of $[^3H]tRNA^{Phe}_{Eid16/17}$. $tRNA^{Phe}$ was reduced with $NaB[^3H]_4$, treated with ethidium bromide, and isolated as described in Materials and Methods. 30 A_{260} units of the modified tRNA were digested with pancreatic RNAase and chromatographed on DEAE-cellulose in the presence of urea as described in Materials and Methods, except that the total gradient volume was 800 ml. A_{260} (—), A_{510} (---), 3H radioactivity (●...●)

$tRNA^{Phe}_{Eid16/17}$ according to the analytical data presented below. A chromatographic profile similar to the one shown in Fig. 1C was obtained for $tRNA^{Phe}_{Eid16/17}$; also in this case only the major fluorescent species was analyzed and used for further experiments.

Oligonucleotide Analyses of $tRNA^{Phe}$ Labeled in the hU Loop. The results reported previously [3] and above (Fig. 2A and 3) already prove that dye incorporation into $tRNA^{Phe}$ depends on excision of a base (wybutine replacement) or modification of bases by $NaBH_4$ reduction (dihydrouracil replacement). $tRNA^{Phe}_{Eid16/17}$ was analyzed in order to (a) establish the extent to which the reduced dihydrouracil residues 16 and/or 17 were replaced by ethidium, (b) determine the amount of ethidium incorporation at the position of 7-methylguanosine which is also reduced by $NaBH_4$

(see above), and (c) verify that there is no replacement of wybutine under the conditions of ethidium incorporation into reduced $tRNA^{Phe}$. The information has been obtained by analyzing the T_1 and pancreatic RNAase digestion products of $tRNA^{Phe}_{Eid16/17}$ and of 3H -labeled $tRNA^{Phe}_{Eid16/17}$. Fig. 4 illustrates the observed cleavage points of the two nucleases in the hU region of $tRNA^{Phe}_{Eid16/17}$. The chromatographic separation of the digestion products is shown in Fig. 2B, C, and 5. A detailed discussion of the analytical data is given in the miniprint at the end of the paper. In brief, the analytical experiments revealed that $tRNA^{Phe}_{Eid16/17}$ as isolated by reversed-phase chromatography (RPC-5; main peak in Fig. 1C) is a mixture of $tRNA^{Phe}_{Eid16}$ ($38 \pm 5\%$), $tRNA^{Phe}_{Eid17}$ ($55 \pm 5\%$), and $tRNA^{Phe}_{Eid16+17}$ ($10 \pm 5\%$). In a small proportion of the molecules ($10 \pm 5\%$) ethidium is also located at the

position of 7-methylguanosine. These numbers are based on the ethidium distribution in the absorbance profile of Fig. 5. No ethidium was found at the wybutine position. In addition, the analyses have shown that ethidium is bound in two different ways (designated B and C as in the case of tRNA^{Phe}_{Eid37}), giving rise to two chromatographically different oligonucleotides for both positions. The tRNA species B and C apparently are not separated by reversed-phase chromatography. For tRNA^{Phe}_{Pri16/17} analytical data were obtained by chromatography of the T₁ RNAase digestion products (described in the miniprint part of the paper) and by the disc electrophoretic analysis of the pancreatic RNAase digestion products. The conclusions are very similar to the ones described for tRNA^{Phe}_{Eid16/17} with the exception that in this case there is no clear separation of two different condensation products. This follows from the observation of a single proflavine-containing dodecanucleotide in disc gel electrophoresis which is probably formed by digestion of tRNA^{Phe}_{Pri17} with pancreatic RNAase in analogy to the digestion of tRNA^{Phe}_{Eid17} (Fig. 4).

Spectroscopic Properties of tRNA^{Phe}-Dye Compounds

The visible absorption and fluorescence spectra of free and tRNA-bound ethidium and proflavine have been measured in the absence and presence of Mg^{2+} (Table 2). The absorption spectrum of ethidium is red-shifted upon binding to the tRNA. The three tRNA^{Phe}-Etd derivatives exhibit somewhat different absorption maxima, the differences being most clearly expressed in the presence of 10 mM Mg^{2+} . The same is true for the emission spectra, which show distinct differences in the extent to which they are blue-shifted relative to the spectrum of the free dye. There is no quantitative correlation of the extents to which the absorption and emission spectra of the three tRNA^{Phe}-Etd derivatives are shifted to the red and the blue respectively. The quantum yield of fluorescence, however, appears to be related to the λ_{max} of emission, since the fluorescence intensity is highest for the species with the most blue-shifted emission spectra. The high quantum yield of tRNA^{Phe}_{Etd37C} relative to that of tRNA^{Phe}_{Etd37B}, which is also clearly shown in Fig. 1A, should be noted. The visible absorption spectra of the two tRNA^{Phe}-Prf derivatives are also substantially red-shifted relative to the free dye, whilst the emission spectra are only slightly changed. In contrast to the observations with ethidium, the proflavine fluorescence is quenched upon incorporation into tRNA.

The spectra of the tRNA^{Phe}-Etd derivatives are clearly influenced by changes of the Mg²⁺ concentration. The effect is most clearly expressed when the fluorescence quantum yields are measured. tRNA^{Phe}_{Etd37B} and tRNA^{Phe}_{Etd16/17} behave similarly: When the Mg²⁺ concentration is increased from below

Table 2. Spectroscopic properties of tRNA^{Phe}-dye compounds

The spectra were measured at 24°C in a buffer containing 10 mM Tris-HCl, pH 7.5, 100 mM KCl, and the indicated concentrations of MgCl₂; the lowest Mg²⁺ concentration was established by the addition of 1.6 mM EDTA to solutions containing 1 mM MgCl₂. The fluorescence was excited at 467 nm with a band width of 4 nm (ethidium) or 1.3 nm (proflavine). The emission data are normalized to the absorbance at 467 nm and are corrected for the wavelength dependence of the sensitivity of the detection system (Materials and Methods).

Compound	Free Mg^{2+}	Absorption (vis λ_{max})	Emission (λ_{max})	Relative fluorescence intensity
	mM	nm		
EtdBr	0-10	480	632	1.0
tRNA ^{Phe} _{Etd37B}	<0.005	510	618	3.2
	1	508	619	2.9
	10	508	620	2.3
tRNA ^{Phe} _{Etd37C}	<0.005	505	616	8.5
	1	500	614	9.1
	10	503	615	7.8
tRNA ^{Phe} _{Etd16/17}	<0.005	505	620	2.5
	1	500	623	2.0
	10	498	624	1.6
Prf	0-10	443	512	1.00
tRNA ^{Phe} _{Prf37}	<0.005	460	515	0.57
	1	458	513	0.62
	10	462	513	0.61
tRNA ^{Phe} _{Prf16/17}	<0.005	462	511	0.12
	1	460	510	0.14
	10	461	510	0.13

0.005 mM to 1 mM and 10 mM the absorption and emission spectra are continuously shifted to the blue and red respectively, and the quantum yields show a continuous decrease. tRNA^{Phc}_{Eid37C} shows a different behaviour: the spectral changes which occur between the lowest Mg²⁺-concentration and 1 mM Mg²⁺ are partially reversed at 10 mM Mg²⁺; concomitantly the quantum yield, which increases upon addition of 1 mM Mg²⁺, decreases again at 10 mM Mg²⁺. This effect was found to be temperature dependent; it was more pronounced at 37 °C and was not found at 10 °C (not shown). Apparently the initial increase of the quantum yield of tRNA^{Phc}_{Eid37C} reflects the Mg²⁺-induced folding of the molecule from some unfolded state which is to be expected at 24 °C and 37 °C when there is practically no Mg²⁺ present. The spectral changes, however, which occur at Mg²⁺ concentrations higher than 1–2 mM have to be ascribed to conformational changes of the folded molecule which, as will be seen in the next paragraph, is in its native state under these conditions. The spectroscopic properties of the tRNA^{Phc}-Prf compounds were changed only slightly upon variation of the Mg²⁺ concentration.

Absorbance measurements with the tRNA^{Phe}-dye compounds purified by reversed-phase chromatogra-

Table 3. Spectroscopic data of the digestion products of tRNA^{Phe}-dye compounds

The spectra have been measured as in Table 2 at 24 °C in 10 mM cacodylate buffer, pH 7.5, 140 mM ammonium sulphate, 100 mM KCl, 1 mM MgCl₂. The digestion mixtures contained in addition T₂ RNAase (15 U/ml), spleen phosphodiesterase (0.12 U/ml), snake venom phosphodiesterase (0.05 U/ml), and alkaline phosphatase (6 U/ml). Digestions were performed at 24 °C until no further change of the fluorescence signal was observed; the usual digestion time was 2 h, tRNA^{Phe}_{Etd37C} had to be digested for 4 h

Compound	Absorption (vis. λ_{max})	Emission (λ_{max})	Relative fluorescence intensity
	nm		
EtdBr	480	632	1.0
tRNA ^{Phe} _{Etd37B}	508	618	2.9
tRNA ^{Phe} _{Etd37B} digested	498	624	1.1
tRNA ^{Phe} _{Etd37C}	500	614	9.1
tRNA ^{Phe} _{Etd37C} digested	495	625	1.3
tRNA ^{Phe} _{Etd16/17}	500	622	2.0
tRNA ^{Phe} _{Etd16/17} digested	495	623	1.2
Prf	443	513	1.00
tRNA ^{Phe} _{Prf37}	458	513	0.62
tRNA ^{Phe} _{Prf37} digested	451	513	1.00
tRNA ^{Phe} _{Prf16/17}	460	510	0.14
tRNA ^{Phe} _{Prf16/17} digested	452	512	0.70

phy (RPC-5) have revealed that the absorption coefficient of the visible absorption of proflavine decreases by about 30% upon binding to the tRNA. The absorption coefficient of bound ethidium, however, was found to be close (within 10%) to the one of the free dye. Thus our earlier assumption [5] of a 30% lower absorption coefficient of ethidium in the tRNA^{Phe}-Etd compounds is no longer valid.

It is known that substitutions at the amino groups of acridine derivatives and related compounds may change their spectroscopic properties. Consequently, we have investigated the extent to which the spectra of the tRNA-bound dyes are influenced by such substitution effects. Because of the lability of the dye-ribosides and their tendency to stick to chromatographic supports, we failed to isolate them from the complete digests of the respective tRNA^{Phe}-dye compounds. Because of these difficulties we have completely digested the tRNA-dye compounds under conditions where the dye ribosides remained intact; this was established by chromatography of the digestion products (see above). The spectra of the digestion mixtures were then measured without isolating the products (Table 3). In all cases the total digestion of the tRNA^{Phe}-dye derivatives shifts the spectra about half the way back towards those of the free dyes. The disappearance of the large quantum yield difference of species B and C of tRNA^{Phe}_{Etd37} upon digestion should be noted.

From these observations it is concluded that the spectroscopic properties of the tRNA-bound dyes are determined to a large extent by the particular local environment at the binding site. This is most clearly seen in the fluorescence intensity data (Table 3). The differences between dye ribosides and free dyes, which are greatest for the absorption spectra, may be due to the substitution at the amino groups of the dyes or to interactions of the dye ribosides with other components of the digestion mixtures. An influence of the ribose substituent on the spectrum of the proflavine riboside is indicated by the observation that an acetic acid treatment of the digestion products of both tRNA^{Phe}_{Prf37} and tRNA^{Phe}_{Prf16/17} restores the absorption spectrum of the free dye.

Stability of the Isomeric Forms of the tRNA^{Phe}-Dye Derivatives

In addition to the rechromatography mentioned above several experiments have been performed in order to establish that the isomeric forms of tRNA^{Phe}_{Etd37} and tRNA^{Phe}_{Prf37} do not interconvert. As judged from their spectroscopic and/or chromatographic properties the isomers were stable during prolonged incubations under the conditions of the aminoacylation assay in the absence or presence of the synthetase. As discussed above the B and C forms of tRNA^{Phe}_{Etd37} upon nuclease digestion give rise to ethidium-containing oligonucleotides which exhibit different chromatographic (Fig. 2 and 5) and electrophoretic (Table 1) properties. The same is true for tRNA^{Phe}_{Etd16/17}. It is concluded that both the B and C forms of the ethidium derivatives and the two forms of the proflavine derivatives are stable isomers.

Activities of tRNA^{Phe}-Dye Compounds

High activity in biochemical assay systems is a prerequisite for the meaningful use of fluorescent tRNA derivatives and probably the most sensitive criterion for the presence of the native conformation of the tRNA molecule. We have, therefore, extended previous studies [5] and have investigated the aminoacylation of the tRNA^{Phe}-dye derivatives in some detail.

Aminoacylation with Phenylalanyl-tRNA Synthetase from Yeast. All tRNA-dye compounds mentioned above could be aminoacylated to at least 80% of the charging level of unmodified tRNA^{Phe}. This activity was maintained also through the reversed-phase column chromatography when the necessary precautions were taken (Materials and Methods). Michaelis-Menten kinetics revealed a K_m of $0.2 \pm 0.05 \mu M$ for tRNA^{Phe} and all derivatives mentioned above. The maximal velocity was lower by approximately 30–50% for the tRNA^{Phe}-dye compounds as compared to unmodified tRNA^{Phe}. In addition, the

Phenylalanine incorporation

Fig. 6. tRNA^{Phe} phenylalanine measurement (100 m the of MethoMg²⁺ meas saturant ii Mg²⁺ of th to inc struct Mg²⁺ maxim. RNA comp Ar tase fr pheny showr [25]. the he corpo vacate hetero the of shown affecte dihydi affecte hydroi the ac the an amino nounc A sma isomer the act caused worthy

UNIVERSITY OF CALIFORNIA, IRVINE

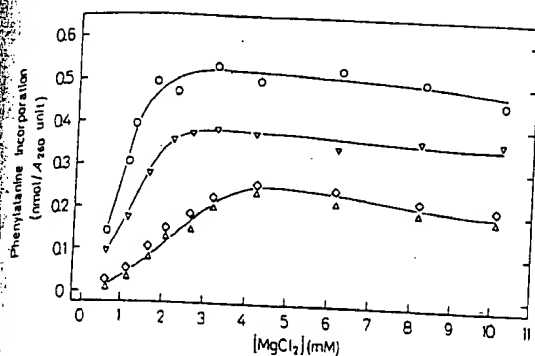


Fig. 6. Mg^{2+} dependence of the aminoacylation of $tRNA^{Phe}$ (O), $tRNA_{Eid16/17}^{Phe}$ (▽), $tRNA_{Eid37B}^{Phe}$ (◇), and $tRNA_{Eid37C}^{Phe}$ (Δ) with phenylalanyl-tRNA synthetase from yeast. Aminoacylation was measured in 0.1-ml assays containing 10 mM Tris-HCl, pH 7.5, 100 mM KCl, 0.1 mM ATP, varying concentrations of $MgCl_2$, and the other components of the aminoacylation assay (Materials and Methods); incubation was for 2 min at 23°C.

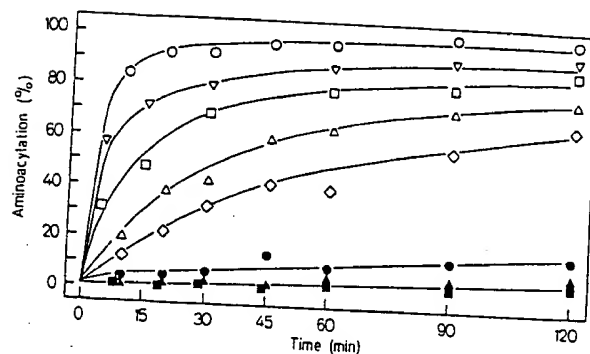


Fig. 7. Aminoacylation of $tRNA^{Phe}$ derivatives with phenylalanyl-tRNA synthetase from *E. coli*. The assay was performed in the presence of 1 M $(NH_4)_2SO_4$ as described previously [25]. In addition to the symbols of Fig. 6 the following symbols are used: $tRNA_{Wyb}^{Phe}$ (●), $tRNA_{Eid37}^{Phe}$ (□), $NaBH_4$ -reduced $tRNA_{Eid37}^{Phe}$ (■) and $tRNA_{Eid37}^{Phe}$ (▲; the mixture of species B and C was used).

DISCUSSION

The location of ethidium or proflavine in the anticodon or hU loops of four $tRNA^{Phe}$ -dye derivatives is established by the results of the structural analyses. As judged from the rather high activities in the homologous and heterologous aminoacylation assay and also in the ribosomal systems from several organisms [10, 26, 27] the insertion of the dyes causes only small alterations of the native $tRNA^{Phe}$ structure. The small shift of the Mg^{2+} optimum of the homologous aminoacylation of $tRNA^{Phe}$ by insertion of ethidium (Fig. 6) should be compared with the extensive shift caused by the excision of wybutine [25]. Accordingly, the direct structural investigation by nuclear magnetic resonance spectroscopy has shown that the insertion of proflavine reverses the substantial changes of the spectrum which are introduced by the excision of wybutine from $tRNA^{Phe}$ [28]. As discussed in the following, the chromatographic and spectroscopic data allow one to develop a rather detailed picture of the chemical structure and the steric arrangement of the dye ribosides in the $tRNA^{Phe}$ -dye compounds.

From the knowledge of sugar-amine condensations [29], the proflavine and ethidium ribosides in the $tRNA^{Phe}$ -dye compounds are expected to be present in the form of ribofuranosides. Such a structure is in keeping with the finding that $NaBH_4$ reduction, which leads to the open-chain ribitol amine derivatives, completely abolishes the chargeability of both $tRNA_{Eid37}^{Phe}$ and $tRNA_{Eid37}^{Phe}$ by phenylalanyl-tRNA synthetase from *E. coli* (Fig. 7). According to the reversed-phase chromatography (RPC-5) profiles, each of the $tRNA^{Phe}$ -dye derivatives is present in two isomeric forms (Fig. 1). No such behaviour was observed with $tRNA^{Phe}$ -hydrazine derivatives [7].

Mg^{2+} dependence of the aminoacylation rate was measured (Fig. 6). A rather low (although still nearly saturating) concentration of ATP (0.1 mM) was present in these experiments in order to minimize the Mg^{2+} buffering effect of ATP. The similar response of the charging rate of $tRNA_{Eid16/17}^{Phe}$ and $tRNA^{Phe}$ to increasing Mg^{2+} concentrations indicates a similar structure of the two tRNAs. A somewhat higher Mg^{2+} concentration was necessary to reach the maximal charging rate of both $tRNA_{Eid37B}^{Phe}$ and $tRNA_{Eid37C}^{Phe}$, indicating some structural differences as compared to unmodified $tRNA^{Phe}$.

Aminoacylation with Phenylalanyl-tRNA Synthetase from *E. coli*. The chargeability of yeast $tRNA^{Phe}$ by phenylalanyl-tRNA synthetase from *E. coli* has been shown to be abolished upon excision of wybutine [25]. We have previously reported that the activity in the heterologous assay is partially restored by the incorporation of proflavine into $tRNA^{Phe}$ at the position vacated by excision of wybutine [5]. The studies of heterologous aminoacylation have been extended to the other $tRNA^{Phe}$ -dye derivatives. The results are shown in Fig. 7. The activity of $tRNA^{Phe}$ is not affected by $NaBH_4$ reduction under conditions where dihydrouracil, wybutine, and 7-methylguanosine are affected (see above). Replacement of reduced dihydrouracil with proflavine or ethidium diminishes the activity only slightly. Substitution of wybutine in the anticodon loop by proflavine lowers the rate of aminoacylation. This effect is somewhat more pronounced when ethidium is substituted for wybutine. A small difference in the activities of the B and C isomers of $tRNA_{Eid37}^{Phe}$ is observed. The complete loss of the activities of $tRNA_{Eid37}^{Phe}$ and $tRNA_{Eid37}^{Phe}$, which is caused by $NaBH_4$ reduction, is particularly noteworthy.

As already discussed in the Results section, there is a number of observations suggesting that the B and C isomers of tRNA^{Phe}_{Etd37} arise from the condensation at the 7-amino and the 2-amino groups of ethidium respectively. There is no direct structural proof, however; the alternative explanation by anomerism appears rather unlikely but cannot be ruled out completely. For the proflavine derivatives of tRNA^{Phe}, on the other hand, it seems clear that α , β anomerism explains the appearance of chromatographically separable isomeric compounds.

The different chromatographic behaviour of the isomeric tRNA^{Phe}-dye compounds most likely reflects differences of the extent to which the hydrophobic dyes are exposed at the surface of the tRNA molecule. The most stable configuration is probably determined by the interactions between the positively charged ring nitrogens of the dyes and a phosphate group of the backbone and between the aromatic systems of dyes and neighboring bases.

The existence of stacking interactions is suggested by comparison of the spectra of the tRNA^{Phe}-dye compounds (Table 2) with those of the dyes intercalated in double-stranded DNA. Accordingly, the absorption and emission spectra were shifted towards the ones of the free dyes when the tRNA-dye compounds are enzymatically digested to the nucleoside level (Table 3). Evidence for stacking interactions of ethidium in the tRNA^{Phe}-Etd derivatives has also been obtained by measurements of the rotational relaxation times which have shown that the dye in both the anticodon and hU loops has little or no freedom to move relative to the tRNA-molecule [9, 30].

The spectroscopic properties of the tRNA^{Phe}-dye compounds are found to be dependent on the position of the dye in the tRNA-molecule (Table 2). The differences are most clearly expressed in the fluorescence properties: the quantum yields of both dyes are higher when the adjacent base is an adenine (anticodon loop) than when it is a guanine (hU loop). These results are in keeping with similar observations which have been reported for the DNA complexes of both proflavine [31, 32] and ethidium [33].

From the data for intercalated ethidium the rather high fluorescence of species C of tRNA^{Phe}_{Etd37} is expected. The comparably low fluorescence of species B may be explained on the basis of a recent hypothesis concerning the fluorescence of intercalated ethidium [34], in which the high fluorescence is ascribed to a shielding of the 2-amino group of ethidium against access of water. Thus the fluorescence data of Table 2 indicate that in the highly fluorescent C isomer the ethidium is bound via the 2-amino group whereas in the much less fluorescent B isomer the 7-amino group has reacted, leaving the 2-amino group free for the access of water. The equally low fluorescence of the ethidium ribosides (Table 3) is consistent with the

interpretation. Thus, the same conclusions with respect to the chemical structure of the tRNA^{Phe}-Etd compounds emerge from the interpretation of the fluorescence and the analytical data.

Variation of the Mg²⁺ concentration in the mM range strongly influence the fluorescence properties of the tRNA^{Phe}-Etd derivatives (Table 2); more detailed investigations [9, 30] have shown that the effects are probably due to Mg²⁺-induced changes of the equilibrium between different conformations of the tRNA^{Phe} molecule. In the same range of Mg²⁺ concentrations the rate of the aminoacylation reaction is found to be maximal (Fig. 6). The observation that modification of the tRNA slightly changes the Mg²⁺ optimum of the reaction suggests that it is influenced by Mg²⁺ binding to the tRNA. Thus the simultaneous occurrence of optimal charging and high flexibility indicates that the ability to exist in alternative conformations is an important feature of the functional design of the tRNA molecule.

We wish to thank H. Aquila and A. Lohmann for expert technical assistance, R. Kientsch and J. Engel for performing some of the digestion experiments, and Prof. A. Böck for repeated generous gifts of phenylalanyl-tRNA synthetase from *E. coli*. The work was supported by Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 51, and Fonds der Chemischen Industrie.

REFERENCES

1. Reines, S. A. & Cantor, C. R. (1974) *Nucleic Acids Res.* 1, 767–786.
2. Yang, C. H. & Söll, D. (1974) *Proc. Natl. Acad. Sci. U.S.A.* 71, 2838–2842.
3. Pingoud, A., Kownatzki, R. & Maass, G. (1977) *Nucleic Acids Res.* 4, 327–338.
4. Schiller, P. W. & Schechter, A. N. (1977) *Nucleic Acids Res.* 4, 2161–2167.
5. Wintermeyer, W. & Zachau, H. G. (1971) *FEBS Lett.* 18, 214–218.
6. Wintermeyer, W. & Zachau, H. G. (1974) *Methods Enzymol.* 29, 667–673.
7. Schleich, H. G., Wintermeyer, W. & Zachau, H. G. (1978) *Nucleic Acids Res.* 5, 1701–1713.
8. Pachmann, U., Cronwall, E., Rigler, R., Hirsch, R., Wintermeyer, W. & Zachau, H. G. (1973) *Eur. J. Biochem.* 39, 265–273.
9. Rigler, R., Ehrenberg, M. & Wintermeyer, W. (1977) *Mol. Biol. Biochem. Biophys.* 24, 219–244.
10. Robertson, J. M., Kahan, M., Wintermeyer, W. & Zachau, H. G. (1977) *Eur. J. Biochem.* 72, 117–125.
11. Fairclough, R. H., Cantor, C. R., Wintermeyer, W. & Zachau, H. G. (1979) *J. Mol. Biol.* in the press.
12. Wintermeyer, W., Robertson, J. M., Weidner, H. & Zachau, H. G. (1979) *tRNA* (Abelson, J., Schimmel, P. & Söll, D., eds) Cold Spring Harbor Laboratory, New York, in the press.
13. Wintermeyer, W. & Zachau, H. G. (1975) *FEBS Lett.* 51, 306–309.
14. Thiebe, R. & Zachau, H. G. (1968) *Eur. J. Biochem.* 5, 546–555.
15. Philippsen, P. & Zachau, H. G. (1972) *Biochim. Biophys. Acta* 277, 523–538.

16. Hirsch, R. & Zachau, H. G. (1976) *Hoppe-Seyler's Z. Physiol. Chem.* 357, 509–526.
17. Haar, F. v. d. (1973) *Eur. J. Biochem.* 34, 84–90.
18. Hanke, T., Barthmann, P., Hennecke, H., Kosakowski, H. M., Jaenicke, R., Holler, E. & Böck, A. (1974) *Eur. J. Biochem.* 43, 601–607.
19. Igo-Kemenes, T. & Zachau, H. G. (1969) *Eur. J. Biochem.* 10, 549–556.
20. Pearson, R. L., Weiss, J. F. & Kelmers, A. D. (1971) *Biochim. Biophys. Acta*, 228, 770–774.
21. White, C. E. & Argauer, R. J. (1970) *Fluorescence Analysis*, Marcel Dekker, New York.
22. Zimmermann, I. & Zimmermann, H. W. (1976) *Z. Naturforsch.* 31c, 656–660.
23. Molinaro, M., Sheiner, L. B., Neelon, F. A. & Cantoni, G. L. (1968) *J. Biol. Chem.* 243, 1277–1282.
24. Wintermeyer, W., Schleich, H. G. & Zachau, H. G. (1978) *Methods Enzymol.* in the press.
25. Thiebe, R. & Zachau, H. G. (1970) *Biochim. Biophys. Acta*, 217, 294–304.
26. Wintermeyer, W. & Zachau, H. G. (1975) *Mol. Biol. (Mosc.)* 9, in Russian: 63–69; in English 49–53.
27. Odom, O. W., Hardesty, B., Wintermeyer, W. & Zachau, H. G. (1975) *Biochim. Biophys. Acta* 378, 159–163.
28. Wong, K. L., Kearns, D. R., Wintermeyer, W. & Zachau, H. G. (1975) *Biochim. Biophys. Acta*, 395, 1–4.
29. Ellis, G. P. & Honeyman, J. (1955) *Adv. Carbohydrate Chem.* 10, 95–168.
30. Ehrenberg, M., Rigler, R. & Wintermeyer, W. (1979) *Biochemistry*, in the press.
31. Pachmann, U. & Rigler, R. (1972) *Exp. Cell Res.* 72, 602–608.
32. Weisblum, B. & Haseth, P. L. de (1972) *Proc. Natl. Acad. Sci. U.S.A.* 69, 629–632.
33. Schreiber, J. P. & Daune, M. P. (1974) *J. Mol. Biol.* 83, 487–501.
34. Olmstedt III, J. & Jearns, D. R. (1977) *Biochemistry*, 16, 3647–3654.

W. Wintermeyer and H. G. Zachau, Institut für Physiologische Chemie, Physikalische Biochemie und Zellbiologie der Ludwig-Maximilians-Universität München, Goethestraße 33, D-8000 München 2, Federal Republic of Germany

FLUORESCENT DERIVATIVES OF YEAST tRNA^{Phe} OLIGONUCLEOTIDE ANALYSES OF HU-LOOP LABELED tRNA^{Phe}-DYE DERIVATIVES:

W. Wintermeyer and H. G. Zachau

Analyses of tRNA^{Phe} Etd16/17

Etd at HU positions 16 and 17. Upon column chromatography of the T1 RNAase digestion products of tRNA^{Phe}Etd16/17 (Fig. 2B), two major (1 and 2) and two minor (3 and 4) peaks of Etd fluorescence were observed. According to the quantitative determination on the basis of Etd absorbance (not shown in Fig. 2B), peaks 1 and 2 together amounted to 85–90 % of the Etd present in the eluate. An analogous analysis of ³H-labeled tRNA^{Phe}Etd16/17, prepared using [³H]NaBH₄ in the reduction step, showed that both peaks 1 and 2 contained ³H-labeled, reduced dihydrouracil. Rechromatography of the radioactive material from peak 1 on DEAE-cellulose in bicarbonate buffer, pH 8, resolved a non-fluorescent, non-radioactive oligonucleotide, presumably C-m²G(2',3')p (1), and two fluorescent, radioactive oligonucleotides. Similarly, the material from peak 2 was resolved into two fluorescent, radioactive compounds upon rechromatography (data not shown). From these results we conclude that peaks 1 and 2 (Fig. 2B) represent the oligonucleotides (E(B), HU(red))p and (E(C), HU(red))p, respectively, which are the products of replacing the reduced dihydrouracils in position 16 or 17 with Etd.

Distribution of Etd between HU positions 16 and 17 as determined by pancreatic RNAase digestion. Pancreatic RNAase does not cleave the 5' phosphoester bond of a nucleotide following an Etd-riboside as shown by the appearance of an Etd containing hexanucleotide upon digestion of tRNA^{Phe}Etd37 (Table 1). The enzyme still cleaves next to reduced dihydrouracil as was shown by digestion experiments with NaBH₄ reduced tRNA^{Phe}, which yielded the same gel electrophoretic oligonucleotide pattern as non-reduced tRNA^{Phe}. However, when the products of pancreatic RNAase digestion of tRNA^{Phe}Etd16/17 were analyzed by polyacrylamide gel electrophoresis, it was found that instead of the expected Etd-containing nonnucleotides, two of three poorly resolved, longer, fluorescent oligonucleotides were formed at the expense of the octanucleotide C-G-A-G-A-G-Cp from the dihydrouridine region (Fig. 4). The electrophoretic mobilities were in the same range as the ones found for the Etd-containing dodecanucleotides from the anticodon region of tRNA^{Phe}Etd37 (Table 1). Apparently, pancreatic RNAase does not significantly cleave the phosphodiester bond between reduced dihydrouridine and an Etd riboside. This leads to the formation of the B- and C-form of a dodecanucleotide carrying Etd at position 17 (II in Fig. 4) in addition to the expected dodecanucleotide carrying Etd at both positions 16 and 17 (III in Fig. 4). In order to perform a more detailed analysis, ³H-labeled tRNA^{Phe}Etd16/17 (see above) was digested with pancreatic RNAase and the products separated on DEAE-cellulose in the presence of urea (Fig. 5). The oligonucleotides containing four or more nucleotides could be characterized by their electrophoretic mobilities in polyacrylamide gels; thus the large radioactive peak at 420 ml, for example, was shown to represent the hexanucleotide Gm-A-A-wy(red)-A-p from the anticodon loop. The elution profile showed two peaks containing Etd and radioactivity (peaks 6 and 7 in Fig. 5), which were eluted just after the octanucleotide (peak at 520 ml). From their electrophoretic mobilities these oligonucleotides were estimated to have a chain length of 12 nucleotides (Table 1). Further proof was obtained by digestion of the material from peaks 6 and 7 with T1 RNAase (Fig. 2C). Besides G(2',3')p, A-Cp, and A-Gp, which were identified by their UV spectra, no peaks containing Etd and radioactivity were observed which corresponded to the two main Etd containing peaks of

Fig. 2B. We conclude that peaks 6 and 7 (Fig. 5) represent the Etd containing dodecanucleotides from tRNA^{Phe}Etd17. From the comparison of dye content and specific radioactivity of peaks 6 and 7, we conclude further that the dodecanucleotide containing Etd at both positions 16 and 17 is also present in peak 6. The smaller Etd containing oligonucleotides of Fig. 5 were rechromatographed on DEAE cellulose in bicarbonate buffer. The elution profiles showed that peaks 1 and 2 each represented one oligonucleotide containing both Etd and radioactive, reduced dihydrouracil. According to this finding and the elution position, peaks 1 and 2 represent the B- and C-form of the Etd containing tetranucleotide A-G-E-HU(red)p expected from pancreatic RNAase digestion of tRNA^{Phe}Etd16 (I in Fig. 4).

The Etd-labeled oligonucleotide from peak 3, which according to rechromatography contained radioactivity, cannot be assigned unambiguously. Probably it is formed by some nonspecific cleavage in the sequence G-G-A-G-A-G-Cp of the dodecanucleotides found in peaks 6 or 7. Preferential nonspecific cleavage in this sequence during digestion of tRNA^{Phe} with pancreatic RNAase has been reported (1).

Etd at the m²G position. Peaks 3 and 4 (Fig. 2B) and peaks 4 and 5 (Fig. 5), which contained 10–15 % of the total Etd absorbance present in the respective elution profile, have not been analyzed further because of the low amount of material. They are eluted as expected for the species B and C of the hexanucleotides E-U-C-m²C-U-Gp (peaks 3 and 4, Fig. 2B) and G-G-A-G-E-Up (peaks 4 and 5, Fig. 5), respectively, which result from the replacement of reduced 7-methylguanine with Etd. The assignment is supported by the observation that the two oligonucleotides did not contain radioactivity when isolated from ³H-labeled tRNA^{Phe}Etd16/17. In addition we found that tRNA^{Phe}, which has been reduced with NaBH₄ under conditions in which only 7-methylguanosine in tRNA^{Phe} was affected, accepted approximately 0.1 mole of Etd per mole of tRNA per hour under the conditions of Fig. 3. This amount compares well with the amount of Etd found in peaks 3 plus 4.

Wybutine is not replaced by Etd. No Etd fluorescence was detected near the elution position of the dodecanucleotide from the anticodon region (last peak in Fig. 2B). A replacement of wybutine with Etd under the conditions of Fig. 3 is therefore excluded.

Analysis of tRNA^{Phe}Etd16/17. The analytical data obtained for tRNA^{Phe}Etd16/17 are similar to the ones described for Etd and will be summarized only briefly. The oligonucleotide pattern after digestion with T1 RNAase was comparable to the profile shown in Fig. 2B. Prf was found in one prominent, heterogeneous peak eluting somewhat later than peak 2 in Fig. 2B and in a rather small peak near the position of peak 1 of Fig. 2B. In addition, two very small peaks near the positions of peaks 3 and 4 were observed, one of which probably represents the product of 7-methylguanine replacement.

The disc electrophoretic analysis of the pancreatic RNAase digestion products showed that a Prf containing dodecanucleotide was formed, the amount of which corresponded to a loss of the octanucleotide from the dihydrouridine region as found for tRNA^{Phe}Etd16/17. Based on these observations and by analogy with the interpretation given for tRNA^{Phe}Etd16/17, we conclude that tRNA^{Phe}Etd16/17 is a mixture of comparable amounts of tRNA^{Phe}Etd16 and tRNA^{Phe}Etd17. The exact distribution of the Prf label between positions 16 and 17 has not been determined.

REFERENCE

1. RajBhandary, U.L., Stuart, A., and Chang, S.H. (1968) *J. Biol. Chem.* 243, 584–591.

[7] A New Method for Attachment of Fluorescent Probes to tRNA¹

By SCOTT A. REINES and LADONNE H. SCHULMAN

Fluorescent probes have been attached to tRNAs by modification of specific minor bases,²⁻⁷ by coupling to the periodate-oxidized 3' terminus,⁸⁻¹¹ through pyrophosphate linkage to the 5' terminus,¹² by reaction with the primary amino group of aminoacyl-tRNA,^{13,14} by replacement of the 3'-terminal adenosine with formycin,¹⁵⁻¹⁷ and by modification of guanine residues.¹⁸ In the present report, we describe a new method for attachment of fluorescent dyes to cytidine residues in tRNA.

Principle

Cytidine and uridine residues in single-stranded regions of nucleic acids are readily modified by addition of sodium bisulfite to the 5,6 double bond of the pyrimidine base.¹⁹ Cytidine-bisulfite adducts undergo deamination by reaction with water and are converted to N⁴-substituted cytidine derivatives by transamination with an appropriate amine. We

¹ This research was supported by grants from the National Institutes of Health (GM 16995) and the American Cancer Society (NP-19). L. H. S. is recipient of an American Cancer Society Faculty Research Award (FRA 129).

² W. Wintermeyer and H. G. Zachau, this series, Vol. 29, p. 667.

³ C. H. Yang and D. Söll, *J. Biochem.* 73, 1243 (1973).

⁴ C. H. Yang and D. Söll, *Biochemistry* 13, 3615 (1974).

⁵ C. H. Yang and D. Söll, *Proc. Natl. Acad. Sci. U.S.A.* 71, 2838 (1974).

⁶ A. Pinguod, R. Kownatzki, and G. Maass, *Nucl. Acids Res.* 4, 327 (1977).

⁷ P. W. Schiller and A. N. Schechter, *Nucl. Acids Res.* 4, 2161 (1977).

⁸ J. E. Churchich, *Biochim. Biophys. Acta* 75, 274 (1963).

⁹ D. B. Millar and R. F. Steiner, *Biochemistry* 9, 2289 (1966).

¹⁰ K. Beardsley and C. R. Cantor, *Proc. Natl. Acad. Sci. U.S.A.* 65, 39 (1970).

¹¹ S. A. Reines and C. R. Cantor, *Nucl. Acids Res.* 1, 767 (1974).

¹² C. H. Yang and D. Söll, *Arch. Biochem. Biophys.* 155, 70 (1973).

¹³ D. C. Lynch and P. R. Schimmel, *Biochemistry* 13, 1841 (1974).

¹⁴ A. E. Johnson, R. H. Fairclough, and C. R. Cantor, in "Nucleic Acid-Protein Recognition" (H. J. Vogel, ed.), p. 469. Academic Press, New York, 1977.

¹⁵ D. C. Ward, E. Reich, and L. Stryer, *J. Biol. Chem.* 244, 1228 (1969).

¹⁶ A. Maelicke, M. Sprinzl, F. von der Haar, T. A. Khwaja, and F. Cramer, *Eur. J. Biochem.* 43, 617 (1974).

¹⁷ S. M. Coutts, D. Riesner, R. Römer, C. R. Rabl, and G. Maass, *Biophys. Chem.* 3, 275 (1975).

¹⁸ L. M. Fink, S. Nishimura, and I. B. Weinstein, *Biochemistry* 9, 496 (1970).

¹⁹ For a review of bisulfite reactions with nucleic acids, see H. Hayatsu, *Prog. Nucl. Acid Res. Mol. Biol.* 16, 75 (1976).

have found that cytidine undergoes a rapid reaction with the bifunctional amine carbohydrazide in the presence of bisulfite, leading to formation of a 4-carbohydrazidocytidine derivative.²⁰ This intermediate is reactive with a variety of amine-specific reagents. The procedure described below is used to attach the intensely fluorescent fluorescein moiety to tRNA by the scheme outlined in Fig. 1.

Materials

Escherichia coli tRNA^{Met}, purified as described before²¹ to a specific activity of 1.9 nmol/A₂₆₀ unit
Yeast tRNA^{Phe}, specific activity 0.95 nmol/A₂₆₀ unit, from Boehringer Mannheim

Crude *E. coli* K12 tRNA, from General Biochemicals
Poly(C), from Miles Laboratories

Fluorescein isothiocyanate (96%) from Aldrich Chemical Co., used without further purification

Carbohydrazide, from Aldrich Chemical Co.

Sodium metabisulfite, grade I, from Sigma Chemical Co.

Sodium sulfite, from Fisher Scientific Co.

Sodium sulfite, ³⁵S-labeled, under nitrogen, 50–200 mCi/mmol, from New England Nuclear Corp.

Dimethyl sulfoxide (DMSO), spectro grade, from Mallinckrodt

Procedures

Modification of tRNAs and Poly(C) with Carbohydrazide in the Presence of Sodium Bisulfite

A solution of 2 M sodium bisulfite, pH 6.0, 1 M carbohydrazide, a 10 mM MgCl₂ is prepared by dissolving 0.63 g of Na₂SO₃, 1.43 g of Na₂S₂O₅, and 0.90 g of carbohydrazide in 10 ml of 10 mM MgCl₂. An ethanol precipitate of RNA is dissolved in this solution to give a final concentration of 20 A₂₆₀/ml. The reaction mixture is incubated at 25° for a given amount of time and the reaction is essentially stopped by addition of 10 volumes of water. The sample is dialyzed overnight at 4° vs 1000 volumes of 0.15 M NaCl, 10 mM Tris-HCl, pH 7.0, and then for 3 hr at 4° vs the same volume of 50 mM NaCl, 10 mM Tris-HCl, pH 7.0. The sample is evaporated at room temperature to a concentration of 20 A₂₆₀/ml and precipitated by addition of 2 volumes of 95% ethanol.

²⁰ S. A. Reines and L. H. Schulman, in preparation.
²¹ L. H. Schulman, *J. Mol. Biol.* 58, 117 (1971).

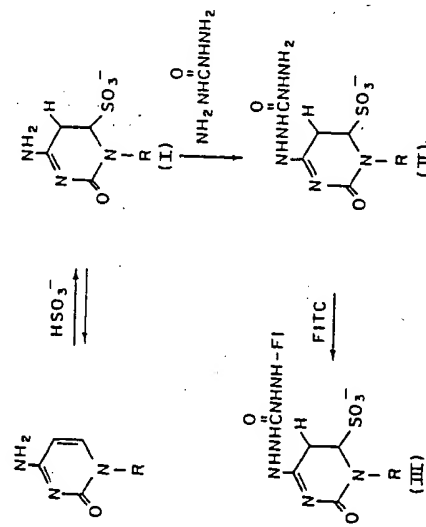


FIG. 1. Sequence of reactions leading to covalent attachment of fluorescein to cytosine derivatives in the presence of bisulfite and carbohydrazide.

A similar procedure is used for modification of poly(C), except that the carbohydrazide concentration is reduced to 0.5 M. Modification of RNAs with [^{35}S]bisulfite is carried out as described above using [^{35}S]Na $_2\text{SO}_3$ instead of unlabeled sodium sulfite.

Determination of the Yield of Carbohydrazide/Bisulfite Adduct II in Poly(C)

Cytidine-bisulfite adducts (I) are unstable and rapidly revert to free cytidine following removal of excess bisulfite. The carbohydrazide-modified adduct (II) is stable for several days at 4°, pH 7, in the absence of free bisulfite. The yield of carbohydrazide/bisulfite adducts can therefore be determined by incorporation of radioactivity into poly(C) from [^{35}S]bisulfite in the presence of carbohydrazide after removal of excess reagents (Fig. 2). This value gives the number of groups in the polymer that can potentially be labeled with dye. There is little or no deamination of cytidine residues under the reaction conditions used.

Uridine-bisulfite adducts are stable at pH 6 and only slowly revert to uridine at neutral pH under the conditions described above. Since many tRNAs contain one or more exposed uridine residues in looped-out regions of the structure, the incorporation of [^{35}S] into tRNAs reflects the amount of uridine-bisulfite adduct formation plus the yield of adduct (II). Uridine adducts in tRNAs can be reversed after dye labeling (see Remarks).

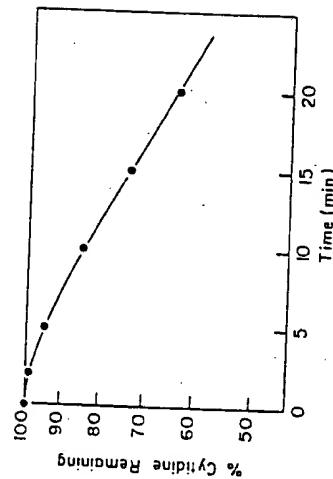


FIG. 2. Rate of modification of cytidine residues in poly(C) in the presence of 2 [^{35}S]bisulfite, pH 6.0, and 0.5 M carbohydrazide at 25°. Formation of adduct (II) was determined by incorporation of [^{35}S] into poly(C) after removal of excess reagents as described in the text. The short lag period corresponds to the time required for formation of an equilibrium concentration of adduct (I).

Labeling of Carbohydrazide/Bisulfite-Modified tRNA and Poly(C) with FITC²²

Conditions for quantitative dye-labeling have been determined using carbohydrazide/bisulfite-modified poly(C) by correlating [^{35}S] incorporation with the yield of covalently attached fluorescein.

The ethanol precipitate of carbohydrazide/bisulfite-modified RNA is dissolved in 0.2 M Tris·HCl, pH 7.0. FITC is dissolved in DMSO just before use to give a concentration of 10 mg/ml. Equal volumes of the RNA and FITC solutions are mixed to give a final reaction mixture containing 20 A $_{260}$ per milliliter of RNA and 5 mg/ml of FITC in 50% DMSO, 0.1 M Tris pH 7.0. The solution is incubated in the dark at 37° for 2 hr, during which time the pH of the solution drops from 7.0 to 5.0 owing to hydrolysis of free FITC. A larger excess of dye should not be used, since the pH may drop below 5 and little or no labeling will occur. One-tenth volume of 4 M NaCl and 3 volumes of 95% ethanol are added to the reaction mixture, the solution is chilled at -20° for 10 min, and the precipitate is collected by centrifugation. The supernatant is discarded, and the RNA is reprecipitated four times from a solution containing 0.1 M Tris·HCl, pH 7.0, 0.5 M NaCl, and 5 mM MgCl $_2$ by addition of 3 volumes of ethanol. The precipitation procedure removes free FITC from the reaction mixture, as indicated by negligible absorption of the

²² Abbreviations: FITC, fluorescein isothiocyanate; FI-tRNA, fluorescein-labeled tRNA; FI, fluorescein thiocarbonyl; DMSO, dimethyl sulfoxide.

final supernatant solution at 495 nm. The free dye is not completely removed by exhaustive dialysis.

Labeling of carbohydrazide/bisulfite-modified RNA is carried out in 50% DMSO in order to drive the reaction to completion within 2 hr by the addition of a large excess of FITC. The solubility of the dye is dependent on the final concentration of both DMSO and buffer in the reaction mixture. Labeling can also be carried out in 0.1 M Tris-HCl, pH 7.0 containing 10% DMSO. Under these conditions, the maximum concentration of FITC that can be used is 2 mg/ml and the rate of labeling is substantially reduced (Fig. 3). The reaction fails to go to completion because of hydrolysis of FITC during the incubation. In order to obtain quantitative labeling at low DMSO concentrations, the modified RNA is incubated in the dark at 37° for 6 hr, precipitated and treated with fresh FITC as before. After three 6-hr incubations at 37° in 0.1 M Tris-HCl, pH 7.0, containing 10% DMSO, 2 mg of FITC per milliliter, the labeling is complete (Fig. 3). If desired, labeling can be carried out at a lower pH using 0.5 M sodium acetate, pH 6.0, containing 50% DMSO and 5 mg of FITC per milliliter. Under these conditions the pH of the reaction is constant during the incubation and labeling is complete within 3 hr (Fig. 3).

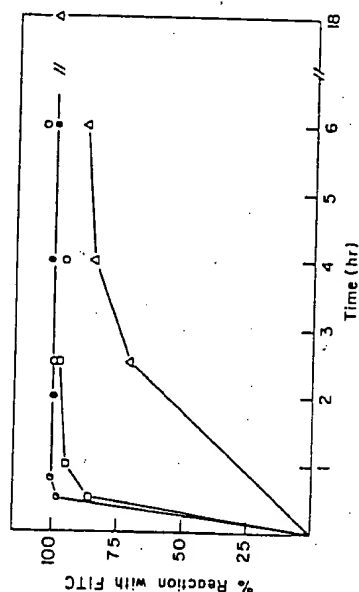


Fig. 3. Rate of reaction of fluorescein isothiocyanate (FITC) with carbohydrazide/bisulfite-modified RNAs. Poly(C) containing 0.1 mol of adduct (II) per mole of CMP: O—, in 0.1 M Tris-HCl, pH 7.0, 50% dimethyl sulfoxide (DMSO), 5 mg FITC/ml; □—, in 0.5 M sodium acetate, pH 6.0, 50% DMSO, 5 mg FITC/ml; Δ—, in 0.1 M Tris-HCl, pH 7.0, 10% DMSO, 2 mg of FITC/ml. *Escherichia coli* tRNA^{phe} containing 1.2 mol of adduct (II) per mole of tRNA: ●—, in 0.1 M Tris-HCl, pH 7.0, 50% DMSO, 5 mg of FITC/ml.

Calculation of Moles of Dye per Mole of RNA

The absorption of RNA-bound fluorescein is lower than that of free dye and is dependent on the number of molecules of dye per molecule of RNA. The exact amount of fluorescein covalently bound to a given RNA is determined after hydrolysis of the sample to mononucleotides and release of free dye by treatment with 0.3 N KOH at 37° for 18 hr. The extinction coefficient of fluorescein varies significantly with pH and dye concentration. After adjusting the solution to pH 7 and 0.1–0.5 A_{495} /ml, an ϵ_{495} of $5.03 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ is used to calculate the yield of free dye.²³ The absorption of fluorescein at 260 nm is subtracted from the total A_{260} in order to obtain the absorption of the hydrolyzed RNA alone.

At low levels of dye labeling, e.g., approximately 1 mol of dye per mole of tRNA, the average extinction coefficient of tRNA-bound fluorescein at 495 nm determined by the above procedure is $4.25 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ in 0.1 M Tris-HCl, pH 7.0, 5 mM MgCl_2 . An extinction coefficient of $5 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ is used for intact tRNA at 260 nm in this buffer. Under these conditions the amount of dye per tRNA is calculated from the equation:

Moles of fluorescein per mole of tRNA

$$= \frac{(A_{495}^{\text{FI-tRNA}} / \epsilon_{495}^{\text{FI-tRNA}})}{(A_{260}^{\text{tRNA}} / \epsilon_{260}^{\text{tRNA}})} \\ = \frac{(A_{495}^{\text{FI-tRNA}} / A_{260}^{\text{tRNA}})}{(A_{495}^{\text{FI-tRNA}} / A_{260}^{\text{tRNA}})} \times 11.8$$

where $A_{260}^{\text{tRNA}} = A_{260}^{\text{FI-tRNA}} - 0.37 \times A_{495}^{\text{FI-tRNA}}$. The ratio of experimentally observed A_{495}/A_{260} is linear with concentration of FI-tRNA up to 0.5 A_{495} /ml, and absorbance measurements are made after adjusting the concentration of tRNA to 0.1–0.5 A_{495} /ml.

Yield of Dye per Mole of tRNA

The yield of dye per mole of tRNA using the procedures described here depends on the rate of modification of a given tRNA with carbohydrazide/bisulfite. This rate is determined by the number and accessibility of cytidine residues in exposed regions of the structure. The yields of dye for several tRNAs under similar reaction conditions are compared in Table I. *E. coli* tRNA^{phe} contains six potentially reactive cytidine residues²⁴ that are modified at different rates. Yeast tRNA^{phe} contains only two exposed cytidines in the 3'-terminal CCA sequence²⁵ and re-

²³ R. P. Tenglerdy and C.-A. Chang, *Anal. Biochem.* 16, 377 (1966).

²⁴ J. P. Goddard and L. H. Schulman, *J. Biol. Chem.* 247, 3864 (1972).

²⁵ D. Rhodes, *J. Mol. Biol.* 94, 449 (1975).

TABLE I
EXTENT OF DYE LABELING FOLLOWING MODIFICATION OF tRNAs WITH
CARBOHYDRAZIDE AND BISULFITE

Sample	Reaction time ^a (min)	Fluorescein/mole tRNA
<i>Escherichia coli</i> tRNA ^{Met}	10	0.99
	20	1.74
Yeast tRNA ^{Phe}	30	1.57
Crude <i>E. coli</i> tRNA	10	1.02
	20	1.62

^a Time of reaction at 25° in 2 M sodium bisulfite, pH 6.0, 1 M carbohydrazide, 10 mM MgCl₂.

quires a longer reaction time to achieve the same extent of dye labeling. An average *E. coli* tRNA is labeled with one dye per mole of tRNA following 10 min of reaction with carbohydrazide/bisulfite as described above.

Optical Properties of Fluorescein-Labeled RNA

The absorption and fluorescence spectra of *E. coli* tRNA^{Met} labeled with 1.5 mol of fluorescein per mole of tRNA are illustrated in Fig. 4. An absorption maximum of 495 nm and fluorescence excitation and emission maxima of 490 nm and 525 nm have been observed at all dye concentrations examined. Increasing the amount of dye per mole of RNA results in a significant decrease in the extinction coefficient at 495 nm and in an increase in the $A_{470}:A_{490}$ ratio of FI-RNA. In addition, a dramatic decrease in fluorescence intensity due to fluorescence quenching from dye-dye interactions is observed, as illustrated in Fig. 5 for fluorescein-labeled poly(C).

Effect of Modifications on Amino Acid Acceptor Activity of tRNAs

The effect of the modification procedures described here on the amino acid acceptor activity of tRNAs depends on the sensitivity of the cognate aminoacyl-tRNA synthetases to structural alterations of exposed cytidine residues in the tRNAs. Such modifications are known to reduce the biological activity of *E. coli* tRNA^{Met}_{26,27}. We have found that carbohy-

²⁶ L. H. Schulman and J. P. Goddard, *J. Biol. Chem.* 248, 1341 (1973).

²⁷ L. H. Schulman and H. Pelka, *Biochemistry* 16, 4256 (1977).

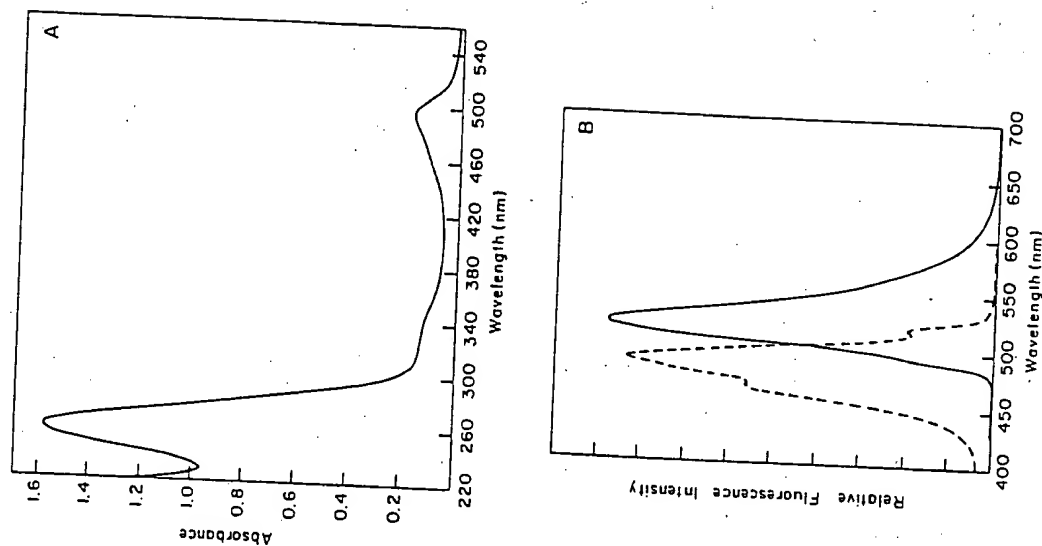


FIG. 4. Optical properties of fluorescein-labeled *Escherichia coli* tRNA^{Met}. (A) Absorption spectrum of *E. coli* tRNA^{Met} containing 1.5 mol of fluorescein per mole of tRNA in 0.1 M Tris-HCl, pH 7.0, 5 mM MgCl₂. (B) ---, Technical fluorescence excitation spectrum (emission at 525 nm); —, emission spectrum (excitation at 490 nm) of the same sample.

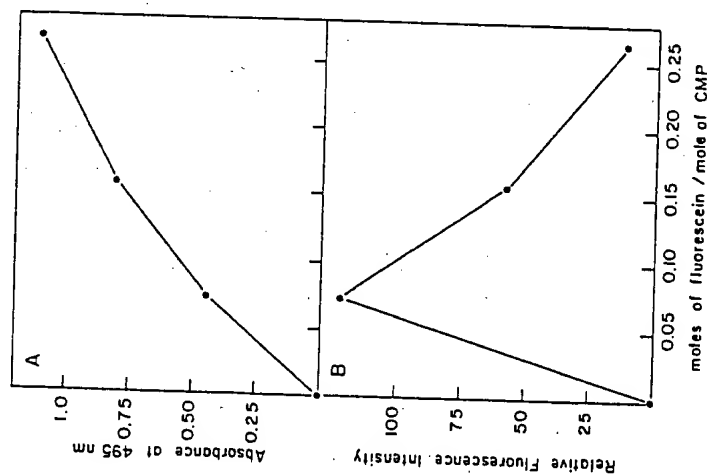


FIG. 5. Effect of dye concentration on the absorption and fluorescence properties of fluorescein-labeled poly(C). (A) Absorbance at 495 nm. (B) Fluorescence emission at 525 nm (excitation at 490 nm). The solvent is 0.1 M Tris-HCl, pH 7.0, 5 mM $MgCl_2$. Fluorescence units are arbitrary.

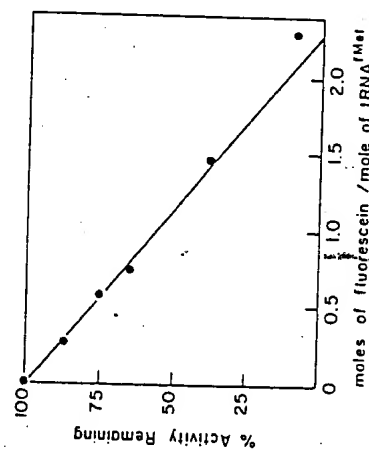


FIG. 6. Effect of the extent of fluorescein labeling of *Escherichia coli* tRNA^{Met} on methionine acceptor activity. Methionine acceptance was measured as described by L. H. Schulman, *J. Mol. Biol.* 58, 117 (1971).

drazide/bisulfite modification of an average of one cytidine per molecule of this tRNA reduces methionine acceptor activity to about 60% of that exhibited by the unmodified tRNA. Attachment of fluorescein has little or no further effect on the activity, and methionine acceptance is a linear function of the number of dye molecules per molecule of tRNA (Fig. 6).

Remarks

Carbohydrazide/bisulfite adduct (II) is less stable than the fluorescein-modified derivative (III). The presence of the unblocked primary amino group in adduct (II) allows this cytidine derivative to undergo intramolecular rearrangements that prevent subsequent reaction with FITC. These side reactions occur rapidly in the presence of acid. After incubation at 4° for 3 hr in acetate buffer, pH 3.5, only 10% of the reactive side chains in carbohydrazide/bisulfite-modified tRNA^{Met} remain available for reaction with FITC. A rapid loss of FITC-reactive groups is also observed above pH 9; however, adduct (II) is relatively stable at neutral pH. Carbohydrazide/bisulfite-modified poly(C) shows a 25% loss of reactivity with FITC following incubation at 25° in 10 mM Tris-HCl, pH 7.0, for 1 month. Slow rearrangement of adduct (II) also occurs when carbohydrazide/bisulfite-modified RNAs are stored as precipitates at -20°. It is therefore recommended that FITC labeling be carried out on freshly prepared samples of modified RNAs. It should also be noted that carbohydrazide/bisulfite-modified tRNAs are potentially capable of forming covalent cross-links to proteins by attack of the ϵ -NH₂ groups of lysine residues at C-4 of the modified pyrimidine base, with displacement of the carbohydrazide side chain.

The fluorescein moiety of dye-labeled RNAs is stable during incubation of the modified RNAs at 37° for 6 hr at pH 5-8. A 10% loss of fluorescein is observed following incubation of dye-labeled poly(C) at 25° in 10 mM Tris-HCl, pH 7.0, for 2 months. Fluorescein-labeled tRNA^{Met} shows no loss of dye after storage as a precipitate for 6 months at -20°, and samples can probably be stored indefinitely in this manner.

The procedures used for carbohydrazide/bisulfite modification of tRNAs lead to formation of uridine-bisulfite adducts in regions of the structure that contain exposed uridine residues. Bisulfite addition to uridine occurs 3-10 times more slowly than formation of adduct (II). Uridine-bisulfite adducts in fluorescein-labeled tRNAs can be completely reversed to unmodified uridine by incubation of the dye-labeled tRNAs in 0.1 M Tris-HCl, pH 9.0, at 37° for 8 hr. These conditions result in a 20% release of fluorescein and a 40% release of free bisulfite from adduct (III).

Other types of amine-specific reagents can be used to attach a variety of fluorescent probes to carbonyl/bisulfite-modified tRNAs. For example, the carbonyl/bisulfite-modified tRNAs. For *N*-hydroxysuccinimide esters (see this volume [8]), and we have covalently attached the naphthoxy moiety to tRNA using the activated ester of dansylglycine and *N*-methylanthranilic acid⁷ can be used to attach these fluorescent probes to the modified tRNAs in a similar manner.

¹⁹ L. H. Schulman, unpublished results.

[8] Attachment of Cross-Linking Reagents to tRNA for Protein Affinity Labeling Studies¹

By ASOK K. SARKAR and LADONNE H. SCHULMAN

A variety of protein affinity-labeling reagents have been attached to tRNAs by covalent linkage to the amino acid moiety of aminoacyl-tRNAs. These peptidyl-tRNA and aminoacyl-tRNA analogs have been used to probe the structure of tRNA binding sites on ribosomes² and to cross-link tRNAs to aminoacyl-tRNA synthetases.³⁻¹⁰ A photoreactive group has also been attached to the periodate-oxidized 3' terminus of tRNA.¹¹ Few methods are presently available for attachment of affinity labels to other regions of tRNA structure. Photolabile azido derivatives have been coupled to the 4-thiouridine residue in several *Escherichia coli*

¹ This research was supported by grants from the National Institutes of Health (GM 16995) and the American Cancer Society (NP-19). L. H. S. is recipient of an American Cancer Society Faculty Research Award (FRA 129).

² For a recent review, see A. E. Johnson, R. H. Fairclough, and C. R. Cantor, in "Nucleic Acid-Protein Recognition" (H. J. Vogel, ed.), p. 469. Academic Press, New York, 1977.

³ C. J. Bruton and B. S. Hartley, *J. Mol. Biol.* 52, 165 (1970).

⁴ D. V. Santi and S. O. Cunliffe, this series, Vol. 29, p. 695.

⁵ D. V. Santi, W. Marchant, and M. Yarus, *Biochem. Biophys. Res. Commun.* 51, 370 (1973).

⁶ O. I. Lavrik and L. Z. Khutoryanskaya, *FEBS Lett.* 39, 287 (1974).

⁷ P. Bartmann, T. Hanke, B. Hammer-Raber, and E. Holler, *Biochem. Biophys. Res. Commun.* 60, 743 (1974).

⁸ I. I. Gorskova and O. I. Lavrik, *FEBS Lett.* 52, 135 (1975).

⁹ V. Z. Akhverdyan, L. L. Kisselev, D. G. Knorre, O. I. Lavrik, and G. A. Nevinsky, *J. Mol. Biol.* 113, 475 (1977).

¹⁰ R. Wetzel and D. Söll, *Nucl. Acids Res.* 4, 1681 (1977).

tRNAs,¹¹⁻¹⁵ and a chemical affinity labeling group has been attached to modified cytidine residues in the 3'-terminal CCA sequence of yeast tRNA^{Phe}.¹⁶ Described herein is a method for coupling a variety of protein affinity-labeling reagents to internal sites in tRNAs.

Principle

Cytidine residues in exposed regions of tRNA structure are chemically modified in the presence of carbonyl/bisulfite and sodium bisulfite to give 4-carbonyl/bisulfite derivatives.¹⁷ The primary amino group of the carbonyl/bisulfite side chain of the modified cytidine residues reacts with *N*-hydroxysuccinimide esters under mild conditions to yield the corresponding amides (Fig. 1). The procedures described below are used to couple several types of protein affinity labeling groups to tRNAs by this general method.

Materials

Escherichia coli tRNA^{Phe}, purified as described before¹⁸ to a specific activity of 1.9 nmol/A₂₆₀ unit.

Crude *E. coli* K12 tRNA, from General Biochemicals

Poly (C), from Miles Laboratories

Bromoacetic acid, from Aldrich Chemical Co.

Succinic acid, from Mallinckrodt

Dicyclohexylcarbodiimide, from Eastman Kodak Co.

N-Hydroxysuccinimide, from Eastman Kodak Co.

Dithiobis (succinimidyl propionate), from Pierce Chemical Co.

Carbonyl/bisulfite, from Aldrich Chemical Co.

Sodium metabisulfite, grade I, from Sigma Chemical Co.

Sodium sulfite, from Fisher Scientific Co.

Fluorescein isothiocyanate, 96%, from Aldrich Chemical Co., used without further purification

Dimethyl sulfoxide, spectro grade, from Mallinckrodt

N,N-Dimethylformamide, spectro grade, from Aldrich Chemical Co.

¹² I. Schwartz and J. Ofengand, *Proc. Natl. Acad. Sci. U.S.A.* 71, 3951 (1974).

¹³ I. Schwartz, E. Gorden, and J. Ofengand, *Biochemistry* 14, 2907 (1975).

¹⁴ V. G. Budker, D. G. Knorre, V. V. Kravchenko, O. I. Lavrik, G. A. Nevinsky, and N. M. Teplova, *FEBS Lett.* 49, 159 (1974).

¹⁵ I. I. Gorskova, D. G. Knorre, O. I. Lavrik, and G. A. Nevinsky, *Nucl. Acids Res.* 3, 1577 (1976).

¹⁶ H. Sternbach, M. Sprinzl, J. B. Hobbs, and F. Cramer, *Enr. J. Biochem.* 67, 215 (1976).

¹⁷ S. A. Reines and L. H. Schulman, this volume [7].

¹⁸ L. H. Schulman and H. Pelka, *J. Biol. Chem.* 252, 814 (1977).

Synthesis of Modified Nucleoside 3',5'-Bisphosphates and Their Incorporation into Oligoribonucleotides with T4 RNA Ligase†

Jorge R. Barrio, Maria del Carmen G. Barrio, Nelson J. Leonard,
Thomas E. England, and Olke C. Uhlenbeck*

ABSTRACT: A simple procedure is described to prepare nucleoside 3'(2'),5'-bisphosphates from the corresponding nucleosides with the use of pyrophosphoryl chloride. This method is rapid, gives nearly quantitative yields and, most importantly, can be used for a variety of nucleosides with base and sugar modifications. Since 3',5'-bisphosphates are donors in the T4 RNA ligase reaction, a single residue can be enzymatically attached to the 3' end of oligoribonucleotides. By these procedures, five different ring-modified nucleosides and one

sugar-modified nucleoside were incorporated onto the 3' end of (Ap)₃C. In two cases, an additional step of synthesis with RNA ligase resulted in the modified nucleotide being located in an internal position in the oligonucleotide. Thus, a general method for the synthesis of oligoribonucleotides containing modified nucleosides is outlined. Since many of the modified nucleosides are fluorescent, oligomers containing them should be useful in a variety of physical and biochemical studies.

The finding that nucleoside 3',5'-bisphosphates are efficient donors in the intermolecular T4 RNA ligase reaction (England & Uhlenbeck, 1978) suggests a convenient method to introduce modified or hypermodified nucleotides into a synthetic oligonucleotide. If a method for the synthesis of the modified 3',5'-bisphosphates were available, RNA ligase could join these molecules onto the 3' end of an oligonucleotide acceptor. After removal of the 3' phosphate the product oligomer containing the modified nucleotide could be used as an acceptor in a subsequent RNA ligase reaction, resulting in a modified residue being placed in an internal position in the sequence. It is likely that substitutions on the base portion of the nucleoside 3',5'-bisphosphate will not affect its efficiency as a donor in the RNA ligase reaction, since RNA ligase shows very little substrate specificity when donors of the type A5'ppX are used in the ATP-independent reaction (England et al., 1977).

We report here that pyrophosphoryl chloride (tetrachloropyrophosphate) (Crofts et al., 1960) can be used for the efficient and general bisphosphorylation of both naturally occurring and highly modified nucleosides to their 3'(2'),5'-bisphosphates and confirm that modified residues can be inserted into the sequence of synthetic oligoribonucleotide with T4 RNA ligase.

Solvents play a critical role in selective phosphorylation with pyrophosphoryl chloride. The reagent has been used successfully in *m*-cresol and in other solvents for the direct and selective phosphorylation of the 5'-hydroxyl group of unprotected nucleosides (Imai et al., 1969; Yoshikawa et al., 1969; Sowa & Ouchi, 1975). Previously, several attempts to phosphorylate primary hydroxyl groups selectively without blocking secondary alcohol functions of nucleosides had not been successful (Barker & Foll, 1957; Ikehara et al., 1963). It was reported that with pyrophosphoryl chloride in the absence of solvent 3'(2'),5'-bisphosphates could be obtained quantitatively (Imai et al., 1969), but the reaction was illustrated only for pGp and

pIp (Honjo et al., 1963). Products that have been obtained using pyrophosphoryl chloride, depending largely upon the experimental conditions, include unsubstituted nucleoside monophosphates (Imai et al., 1969), nucleoside 3'(2'),5'-bisphosphates (Honjo et al., 1963; Simoncsits & Tomasz, 1974; Simoncsits et al., 1975), nucleoside cyclic 2',3'-phosphate 5'-phosphate (Simoncsits & Tomasz, 1975), and highly phosphorylated compounds (Tomasz & Simoncsits, 1975). We have developed conditions for obtaining various 3'(2'),5'-bisphosphates under which the pyrophosphoryl chloride reaction, followed by an extremely simple work-up, becomes both efficient and generally applicable. Moreover, the mixtures of pure bisphosphates can be used directly with the T4-induced RNA ligase since the 3',5' component of the mixture is the substrate and the 2',5' component is neither a substrate nor an inhibitor (England & Uhlenbeck, 1978).

Experimental Section

Chemicals. Adenosine, AMP, pA2'p, pA3'p, and 2'-deoxycytidine were obtained from Sigma Chemical Co.; 2'-O-methylcytidine, 3'-O-methylcytidine, and pG3'p were from P-L Biochemicals. *lin*-Benzoadenosine was prepared by the method of Leonard et al. (1976), except that the deblocking of the sugar group after ribosidation was better carried out with ethanolic ammonia at room temperature for 24 h. Displacement of the methylthio by an amino group to afford *lin*-benzoadenosine is best accomplished at 150 °C during 24 h. The

† Abbreviations used: one letter abbreviations for oligonucleotides and nucleoside 3',5'-bisphosphates will be used (such as: pA3'p, adenosine 3',5'-bisphosphate; pA2'p, adenosine 2',5'-bisphosphate; pAp, a mixture of the two isomers); methylation of the ribose hydroxyl is indicated by the suffix "m" (so pC3'm2'p is 3'-O-methylcytidine 2',5'-bisphosphate); ϵ , etheno (so that ϵ C is 3,N⁴-ethenocytidine or 5,6-dihydro-5-oxo-6- β -D-ribofuranosylimidazo[1,2-c]pyrimidine (Secrist et al., 1972; Barrio et al., 1972); ϵ A is 1,N⁶-ethenoadenosine or 3- β -D-ribofuranosyl[2,1-*i*]purine (Secrist et al., 1972); ϵ G is 1,N²-ethenoguanosine or 5,9-dihydro-9-oxo-3- β -D-ribofuranosylimidazo[1,2-*a*]purine (Sattang et al., 1977)); μ G, 1,N²-(2-methylallylidene)guanosine; *lin*A, *lin*-benzoadenosine, which is the trivial name for 1-(β -D-ribofuranosyl)-8-aminoimidazo[4,5-*g*]quinazoline (Leonard et al., 1976); Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

† From the Departments of Chemistry and Biochemistry, School of Chemical Sciences, University of Illinois, Urbana, Illinois 61801. Received December 5, 1977. This work was supported by Research Grants GM-05829 and GM-19059 from the National Institutes of Health. One of us (O.C.U.) is a National Institutes of Health Research Career Development Awardee.

total yield after these two steps was nearly quantitative. 3',N⁶-Ethenocytidine was prepared by the method of Barrio et al. (1972, 1976). *lin*-Benzo-AMP was prepared by the method of Scopes et al. (1977); 1,N²-ethenoguanosine, by the method of Sattangi et al. (1977). Pyrophosphoryl chloride was best prepared by reaction of phosphoric oxide, phosphorus trichloride, and chlorine in carbon tetrachloride following the method of Crofts et al. (1960). The compound is stable for at least 1 year when stored desiccated at -20 °C. Thin-layer chromatography was performed on Brinkman cellulose F plates using isobutyric acid:NH₄OH:H₂O, 75:1:24. Nucleotides were visualized with ultraviolet light.

Apparatus. Ultraviolet absorption spectra were obtained on a Beckman Acta M VI spectrophotometer. ³¹P nuclear magnetic resonance spectra were obtained on a Varian Associates XL-100-15 NMR system equipped with a Digital NMR-3 data system, operating at 40.5 MHz for ³¹P and 100 MHz for ¹H. Broadband proton decoupling centered at about δ 4.0 was used for proton decoupled phosphorus spectra. Deuterium from the D₂O solvent was used for field/frequency stabilization. Phosphoric acid (85%) in a concentric capillary (2-mm O.D.) was used as primary ³¹P reference. All spectra were obtained by the Fourier transform technique using 16K data points and a 2500-Hz bandwidth.

Enzymes and Enzyme Assays. The source of materials, including the ³H-labeled oligonucleotide acceptor, (Ap)₃C, and the T4 RNA ligase as well as the procedures for running the reaction and analyzing the products are described in detail in the preceding paper (England & Uhlenbeck, 1978). For the evaluation of a modified pNp as a donor, the 30-μL reaction mixture contained 0.1 mM [Cyd-³H](Ap)₃C (330 Ci/mol), 0.2 mM pNp, 0.5 mM ATP, 50 mM Hepes (pH 8.3), 20 mM MgCl₂, 3.3 mM dithiothreitol, 10 μg/mL serum albumin, and various concentrations of T4 RNA ligase. After 60 min at 37 °C, the reaction mixtures were spotted on Whatman 3MM paper and a descending chromatogram was run in 60:40 (v/v) 1 M ammonium acetate:ethanol. The yield of the slower moving (Ap)₃CpNp product spot was calculated as the percentage of ³H label migrating at that position.

The products were characterized as having had a single modified nucleotide added to (Ap)₃C by the series of enzymatic degradations used to identify (Ap)₃CpAp in the preceding paper (England & Uhlenbeck, 1978). Since no ³²P label was present in the donor, only the ³H label in the cytidine can be detected. However, in many cases the nucleotide added is fluorescent and can be seen on the paper chromatogram, thus aiding identification.

(Ap)₃CpεA and (Ap)₃CpC2'm were prepared in slightly larger amounts in order to test their effectiveness as acceptors. Each reaction contained 0.25 mM [Cyd-³H](Ap)₃C (285 Ci/mol), 0.5 mM pNp, 0.5 mM ATP, and 250 U/mL RNA ligase in the same buffer used above. After 60 min at 37 °C, the products (Ap)₃CpNp were purified by paper chromatography as above (yield 96% in both cases). After elution from the paper, a portion of each (Ap)₃CpNp was treated with alkaline phosphatase to form the (Ap)₃CpN, repurified by paper chromatography, eluted, and desalted on Bio-Gel P2 (England & Uhlenbeck, 1978).

The additions of pAp to (Ap)₃CpεA and to (Ap)₃CpC2'm were carried out in an analogous manner. Each reaction contained 10–20 μM [Cyd-³H](Ap)₃CpN (285 Ci/mol), 90 μM [5'-³²P]pA3'p (3 Ci/mol), 330 μM ATP, and 250 U/mL RNA ligase in the same buffer used above. After 18 h at 4 °C, the reaction mixtures were analyzed by paper chromatography as

oxide (0.2 mmol) and pyrophosphoryl chloride (504 mg, 2 mmol) was stirred at -10 to -15 °C. After 4–5 h the reaction was quenched by the rapid addition of ice and, immediately thereafter, a chilled solution of 0.5 M triethylammonium bicarbonate, pH 8.0. The colorless solution was evaporated to dryness under vacuum at 20 °C. The residue was dissolved and evaporated several times with 10-mL portions of methanol to remove the excess triethylammonium bicarbonate and was then chromatographed on a column of DEAE-cellulose (2.5 × 40 cm) with a linear gradient of 0.05 to 0.4 M triethylammonium bicarbonate, pH 8.0. The fractions containing the nucleoside bisphosphate were pooled and evaporated to dryness at 20 °C as indicated above, giving 85–95% of an unresolved mixture of pure nucleoside 2',5'- and 3',5'-bisphosphates as judged by ³¹P NMR.

1,N⁶-Ethenoadenosine 3',5'-bisphosphate (pεA3'p) was prepared by chloroacetaldehyde modification of pA3'p (Barrio et al., 1972). When the reaction was complete, as judged by thin-layer chromatography, the solvent was evaporated to dryness under vacuum at 20 °C. The residue was chromatographed as indicated above in the general preparation of pNp's (yield: 90%).

1,N²-(2-Methylallylidene)guanosine 3',5'-bisphosphate (pμG3'p) was prepared by modification of pG3'p with methylmalonaldehyde at pH 4.2 (0.1 M NaOAc buffer) (Möschel & Leonard, 1976). Chromatographic purification was carried out as indicated above for other pNp's (yield: 50%). Thus, in these representative cases, it was satisfactory to modify the 3',5'-bisphosphates rather than to phosphorylate the modified nucleosides.

Results and Discussion

Chemistry. Nucleoside 3'(2'),5'-bisphosphates were obtained selectively by reaction of each unprotected nucleoside with pyrophosphoryl chloride at -10 to -15 °C for several hours, rapid hydrolysis by means of ice and triethylammonium bicarbonate, and column chromatography on DEAE-cellulose with a linear gradient of triethylammonium bicarbonate. *The nucleoside 3'(2'),5'-bisphosphates were produced almost exclusively under the conditions described.* They were identified by their spectroscopic, chromatographic, and enzymatic properties, and in representative cases by comparison with authentic samples. Under the conditions we employed, by-products were limited to very small amounts of either 5'-monophosphates or higher phosphorylated products. At least two conditions proved to be critical for efficient reaction: (a) purity of the pyrophosphoryl chloride, which decomposes readily when maintained at room temperature for several days; and (b) temperature of the reaction (Honjo et al., 1963; Tomasz & Simoncsits, 1975).

It is unclear why the pyrophosphoryl chloride method of bisphosphorylation, which was introduced in 1963 for the synthesis of pGp and plp, has not received more attention. Limited cognizance of the first report (Honjo et al., 1963) or the multiple manipulations described for purification may have contributed. In any case, the presently described directions have considerably simplified and generalized the procedure. For example, 2'-deoxycytidine could be phosphorylated to pdCp in high yield and with little hydrolysis of the glycosidic bond. Other 3'(2'),5'-bisphosphates produced by this method are included in Table I, and one may safely predict extension of the method for obtaining additional deoxyribonucleoside-3',5'-bisphosphates and modified ribonucleoside 3'(2'),5'-bisphosphates as well. The procedure also overcomes the

TABLE I. ^{31}P NMR Data^a

Compound	Chemical shifts ^b		
	^{31}P (ppm)	2'-P	5'-P
pA3'p	4.175		3.902
pA2'p		3.696	3.962
plinA3'p	4.077		3.865
plinA2'p		3.475	3.865
pdCp	3.287		3.837
pC2'm3'p	3.399		3.762
pC3'm2'p		3.475	3.837
peC3'p	4.049		3.827
peC2'p		3.721	3.916
peG3'p	4.180		3.878
peG2'p		3.566	3.924
AMP			3.882
lin-BenzoAMP			3.954

^a Spectra recorded in D_2O , EDTA (0.002 M), adjusted to pH ~10 by addition of $\text{Me}_4\text{N}^+\text{OH}^-$. ^b Chemical shifts for proton-decoupled phosphorus signals are expressed in ppm downfield from external 85% H_3PO_4 capillary. In the nondecoupled ^{31}P NMR spectra the 5'-phosphate ^{31}P resonance displays a very characteristic split triplet. The two H_5 protons account for the triplet structure of the phosphate ^{31}P signal. A long-range $^4J_{\text{PH}}$ coupling to the sugar ring H_4 proton is also observed. 3'- and 2'-phosphates ^{31}P show characteristic doublets due to couplings of phosphorus to vicinal protons ($^3J_{\text{PH}}$ and $^3J_{\text{PH}'}$, respectively). Long-range $^3J_{\text{PH}}$ coupling constants are also detected (Cozzzone & Jardetzky, 1976b). ^c In the nondecoupled ^{31}P spectra of pA3'p recorded at 40.48 MHz in the Fourier mode, 5'-P is reported as 0.212 ppm upfield from 3'-P (Lee & Sarma, 1975).

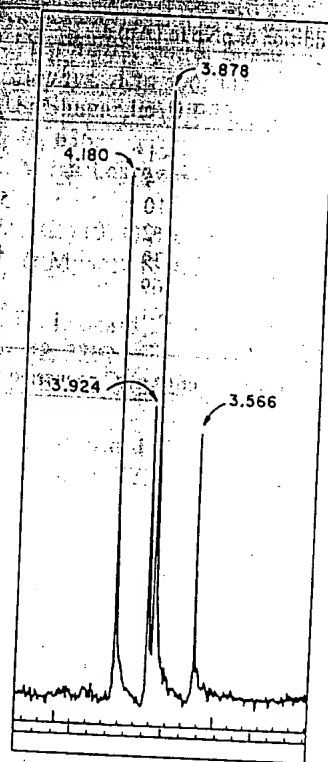


FIGURE 1: Representative proton-decoupled ^{31}P NMR of a mixture of pure nucleoside 3'(2'),5'- and 2',5'-bisphosphates (in this case, 1,N²-ethenoguanosine 3'(2'),5'-bisphosphate) obtained after phosphorylation and chromatographic purification of the products (see Experimental Section and Table I for full details).

preparation of nucleoside 3'(2'),5'-bisphosphates. These include direct phosphorylation of nucleosides with either dibenzyl phosphorochloridate (Moffatt & Khorana, 1961; Dekker et al., 1953; Cramer et al., 1957), phosphorous oxychloride in triethyl phosphate (Morelli & Benatti, 1974), or *N*-phosphoryl-*N*'-methylimidazolium salts (Takaku et al., 1973). The available enzymatic methods for preparation of 3',5'-bisphosphates include hydrolysis of RNA with venom exonuclease (Richards & Laskowski, 1969) or phosphorylation of 3'-monophosphates by polynucleotide kinase (Richardson, 1971). However, the enzymatic production of various modified pNp's could be limited either by their availability in RNA or the substrate specificity of polynucleotide kinase. Thus, the use of pyrophosphoryl chloride for the preparation of unmodified and modified nucleoside 3'(2'),5'-bisphosphates is presently the most convenient available procedure. The method as described here has also demonstrated promise for the bisphosphorylation of unprotected dinucleoside 3',5'-phosphates. When these are subjected to reaction with pyrophosphoryl chloride at -20°C , 3'(2'),5'-bisphosphorylation is the main reaction, and the products are obtained in acceptable yields. Investigation of this route to pNpNp compounds is continuing.

Mechanisms for the bisphosphorylation reaction with pyrophosphoryl chloride have been established and nucleoside cyclic 2',3'-chlorophosphate 5'-dichlorophosphates have been implicated as intermediates (Tomasz & Simoncsits, 1975). We observed a $65 \pm 5\%$ to $35 \pm 5\%$ ratio of pN3'p to pN2'p in all cases where unsubstituted ribonucleosides were submitted to phosphorylation and work-up conditions described in the Experimental Section. The composition of such a mixture is dictated either by stereoselective nucleophilic attack by water on a cyclic 2',3'-phosphate intermediate (Westheimer, 1968; Tomasz & Simoncsits, 1975) or by a regioselective phosphorylation step (Lippert, 1968; Cozzzone & Jardetzky, 1976a).

^{31}P NMR spectroscopy is a simple and powerful tool for the characterization of nucleoside 3'(2'),5'-bisphosphates (Table I). Examination of the proton undecoupled ^{31}P spectra of nucleoside 5'-monophosphates and nucleoside 3'(2'),5'-bisphosphates reveals the striking feature that the 5'-P signals experience very little change from an average value of δ 3.88, downfield from 85% H_3PO_4 , among the compounds listed. Accumulated evidence indicates that the most favored conformation of a ribonucleoside 5'-phosphate is anti at N-C(1') and gauche-gauche along C(4')-C(5'). These are little affected by the addition of a 3'-phosphate, except that this group exhibits greater flexibility than the 5'-phosphate. The ribofuranose ring is in rapid equilibrium between ^2E [C(2')-endo] and ^3E [C(3')-endo] conformations (Sundaralingam, 1969; Remin & Shugar, 1972; Olson & Flory, 1972; Sarma & Myott, 1973; Sundaralingam, 1973; Yathindra & Sundaralingam, 1973a,b; Altona & Sundaralingam, 1973; Lee & Sarma, 1974, 1975; Prusiner et al., 1974; Davies & Danyluk, 1974, 1975; Lapper & Smith, 1975; Evans et al., 1975).

With the signal responsible for the 5'-P nearly constant, the 3'-P and 2'-P signals can be assigned readily when the ribonucleoside 3',5'- and 2',5'-bisphosphates are present in different proportions (Figure 1). The 2'-P signals are consistently upfield from the 5'-P resonance. The 3'-P signals are shifted downfield by ca. 0.20 ppm from the 5'-P average position, a result of the 3'-P environment and the equilibrium among the rotamers, $^2\text{E} \rightleftharpoons ^3\text{E}$: trans, gauche (g^-) and gauche (g^+) about C(3')-O(3'), available to the 3'-phosphate group (Lee & Sarma, 1975). Methylation of the 2'-hydroxyl (pC2'm3'p) or its elimination (pdCp) results in a shielding effect on the 3'-phosphate ^{31}P resonance (Table I). In both cases, 2'-OH interaction is removed (Cozzzone & Jardetzky, 1976a) and shifts in the equilibria of the 3'-phosphate

TABLE II. Single Addition of pNp to (Ap)₃C.

pNp	RNA ligase concentration					
	7 U/mL	35 U/mL	70 U/mL	105 U/mL	245 U/mL	350 U/mL
pμG3'p	<1 ^a	30	45	54	72	75
pεGp	4	5	9	14	25	40
pεA3'p	10	51	79	88	94	94
p ϵ linAp	12	34	54	65	91	94
pεCp	39	87	94	97	96	96
pC2'm3'p	69	92	95	95	95	95
pC3'm2'p	<1	<1	<1	<1	<1	<1
pdCp	25	72	94	98	98	98

^a Yields are given as percent of (Ap)₃C converted to (Ap)₃CpNp.

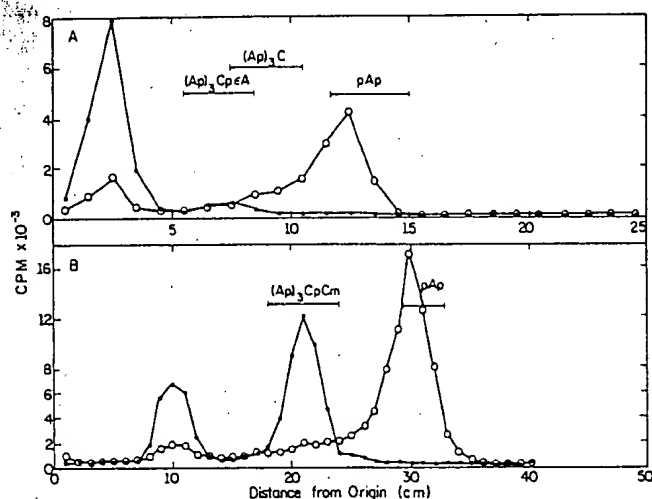


FIGURE 2. Paper chromatographic analysis of the addition of [5'-³²P]pAp to [Cyd-³H](Ap)₃CεA (A) and (Ap)₃CpC2'm (B). Open circles are ³²P radioactivity; closed circles are ³H radioactivity.

relaxation of the phosphorus signal is allowed, the ³¹P relative chemical shifts are useful in structure assignment and in the quantitative estimation of proportions in mixtures such as those encountered in the present synthesis of 3'(2'),5'-bisphosphates.

Enzymology. Seven modified nucleoside 3'(2'),5'-bisphosphates were tested for their ability to act as donors with [Cyd-³H](Ap)₃C acceptor and T4 RNA ligase (Table II). A twofold excess of donor to acceptor was maintained in each reaction in order to ensure that the 3' isomer was present in sufficient quantity. The nucleoside 2',5'-bisphosphates do not affect the ligation reaction as they are neither substrates nor competitive inhibitors of the RNA ligase reaction (England & Uhlenbeck, 1978). The lack of activity observed for pC3'm2'p in Table II supports this conclusion. As can also be seen in Table II, all six of the modified pNp's with a 3' phosphate were active donors and modified oligomers of the type (Ap)₃CpNp were obtained in excellent yields. The identity of these slower moving products was confirmed by digestion of each product with ribonuclease A to give radiolabel which comigrated with (Ap)₃Cp upon analysis by descending paper chromatography and ribonuclease A plus alkaline phosphatase to convert the product back to ³H-labeled (Ap)₃C. Also, in most cases the fluorescence of the modified oligonucleotide could be detected by examining the paper chromatogram in ultraviolet light.

Although the varying proportions of 3' and 2' isomers prevent detailed quantitative comparisons, it is evident that the modified nucleoside bisphosphates are nearly as good sub-

strates as (Ap)₃C (England & Uhlenbeck, 1978). This observation is consistent with the remarkable lack of specificity of RNA ligase that was noted when adenylylated pyrophosphates were used as donors (England et al., 1977).

Examples of the addition of pAp to oligonucleotides containing modified nucleosides are shown in Figure 2. In the upper panel the reaction with (Ap)₃CpεA as the acceptor is analyzed. Nearly 85% of the ³H label in the acceptor is converted to a doubly labeled slower moving product, (Ap)₃CpεApAp. Since the donor [5'-³²P]pAp is in excess, a lesser fraction of the ³²P label is found in the product. In the lower panel, (Ap)₃CpC2'mpAp is obtained in 36% yield under identical reaction conditions for the acceptor (Ap)₃CpC2'm. The lower yield in the latter case strengthens the observation that the reactivity of an acceptor in the RNA ligase reaction is determined by its base composition near the 3' end (England & Uhlenbeck, 1978). In each case, the products were identified by the resistance of ³²P radiolabel to alkaline phosphatase and the production of the expected nucleoside monophosphates [Cyd-³H]Cp and [3'-³²P]εAp or [3'-³²P]C2'm3'p, respectively, upon hydrolysis with spleen phosphodiesterase. Thus, modified nucleosides can be inserted effectively into internal positions in an oligonucleotide sequence.

Our preparation of nucleoside 3'(2'),5'-bisphosphates from the corresponding nucleosides is sufficiently general that a wide variety of modified and hypermodified nucleotides can be obtained. Since the modified bisphosphates are substrates in the RNA ligase reaction, a general method is now available for the synthesis of oligoribonucleotides containing modified bases at specific positions in the sequences and experiments can be designed to clarify the structural and functional roles of modified nucleotides in RNA.

Acknowledgments

We thank Ms. Monique Hinterberger for the preparation of pμGp.

References

- Altona, C., & Sundaralingam, M. (1973) *J. Am. Chem. Soc.* 95, 2333.
- Barker, G. R., & Foll, G. E. (1957) *J. Chem. Soc.* 3798.
- Barrio, J. R., Secrist, J. A., III, & Leonard, N. J. (1972) *Biochem. Biophys. Res. Commun.* 46, 597.
- Barrio, J. R., Sattangi, P. D., Gruber, B. A., Dammann, L. G., & Leonard, N. J. (1976) *J. Am. Chem. Soc.* 98, 7408.
- Cozzone, P. J., & Jardetzky, O. (1976a) *Biochemistry* 15, 4853.
- Cozzone, P. J., & Jardetzky, O. (1976b) *Biochemistry* 15, 4860.

- J. Chem. Soc.*, 3297.
Grotts, P. C., Downie, I. M., & Heslop, R. B. (1960) *J. Chem. Soc.*, 3673.
Davies, D. B., & Danyluck, S. S. (1974) *Biochemistry* 13, 4417.
Davies, D. B., & Danyluck, S. S. (1975) *Biochemistry* 14, 543.
Dekker, C. A., Michelson, A. M., & Todd, A. R. (1953) *J. Chem. Soc.*, 947.
England, T. E., & Uhlenbeck, O. C. (1978) *Biochemistry* 17 (preceding paper in this issue).
England, T. E., Gumpfort, R. I., & Uhlenbeck, O. C. (1977) *Proc. Natl. Acad. Sci. U.S.A.* 74, 4839.
Evans, F. E., Lee, C. H., & Sarma, R. H. (1975) *Biochem. Biophys. Res. Commun.* 63, 106.
Gorenstein, D. G. (1975) *J. Am. Chem. Soc.* 97, 898.
Gruber, W., & Lynen, F. (1962) *Justus Liebigs Ann. Chem.* 659, 139.
Honjo, M., Imai, K., Furukawa, Y., Morijama, H., Yasumatsu, K., & Imada, A., (1963) *Takeda Kenyusikko Nempo* 22, 47.
Ikehara, M., Ohtsuka, E., & Kodama, Y. (1963) *Chem. Pharm. Bull. (Tokyo)* 11, 1456.
Imai, K., Fujii, S., Takanohashi, K., Furukawa, Y., Masuda, T., & Honjo, M. (1969) *J. Org. Chem.* 34, 1547.
Lapper, R. D., & Smith, I. C. P. (1975) *J. Am. Chem. Soc.* 95, 2880.
Lee, C. H., & Sarma, R. H. (1974) *FEBS Lett.* 43, 271.
Lee, C. H., & Sarma, R. H. (1975) *J. Am. Chem. Soc.* 97, 1225.
Leonard, N. J., Sprecker, M. A., & Morrice, A. G. (1976) *J. Am. Chem. Soc.* 98, 3987.
Lynen, F. (1958) *Proc. 4th Int. Congr. Biochem.* 11, 230.
Moffatt, J. G., & Khorana, H. G. (1961) *J. Am. Chem. Soc.* 83, 663.
Morelli, A., & Benatti, U. (1974) *Ital. J. Biochem.* 23, 279.
Moschel, R. C., & Leonard, N. J. (1976) *J. Org. Chem.* 41, 294.
Olson, W. K., & Flory, P. J. (1972) *Biopolymers* 11, 25.
Prusiner, P., Yathindra, N., & Sundaralingam, M. (1974) *Biochim. Biophys. Acta* 366, 115.
Remin, M., & Shugar, D. (1972) *Biochem. Biophys. Res. Commun.* 48, 636.
Richards, G. M., & Laskowski Sr., M. (1969) *Biochemistry* 8, 4858.
Richardson, C. C. (1971) *Proced. Nucleic Acid Res.* 2, 815.
Sarma, R. H., & Mynott, R. J. (1973) *J. Am. Chem. Soc.* 95, 1641.
Sattangi, P. D., Leonard, N. J., & Frihart, C. R. (1977) *J. Org. Chem.* 42, 3292.
Scopes, D. J. C., Barrio, J. R., & Leonard, N. J. (1977) *Science* 195, 296.
Secrist, J. A., III, Barrio, J. R., Leonard, N. J., & Weber, G. (1972) *Biochemistry* 11, 3499.
Simoncsits, A., & Tomasz, J. (1974) *Biochim. Biophys. Acta* 340, 509.
Simoncsits, A., & Tomasz, J. (1975) *Biochim. Biophys. Acta* 395, 74.
Simoncsits, A., Tomasz, J., & Allende, J. E. (1975) *Nucleic Acids Res.* 2, 257.
Sowa, T., & Ouchi, S. (1975) *Bull. Chem. Soc., Jpn.* 48, 2084.
Sundaralingam, M. (1969) *Biopolymers* 7, 821.
Sundaralingam, M. (1973) *Proc. Jerusalem Symp.*, 5th, 381.
Takaku, H., Yoshifusa, S., & Hidetoshi, O. (1973) *Chem. Pharm. Bull. (Tokyo)* 21, 1844.
Tomasz, J., & Simoncsits, A. (1975) *J. Carbohydr. Nucleosides Nucleotides* 2, 315.
Westheimer, F. H. (1968) *Acc. Chem. Res.* 1, 70.
Yathindra, N., & Sundaralingam, M. (1973a) *Biopolymers* 12, 297.
Yathindra, N., & Sundaralingam, M. (1973b) *Biopolymers* 12, 2261.
Yoshikawa, M., Kato, T., & Takenishi, T. (1969) *Bull. Chem. Soc. Jpn.* 42, 3505.

Pyrene Derivatives as Fluorescent Probes of Conformation Near the 3' Termini of Polyribonucleotides

PAUL KOENIG, SCOTT A. REINES, and CHARLES R. CANTOR,
*Departments of Chemistry and Biological Sciences, Columbia
University, New York, New York 10027*

Synopsis

When pyrene butyric acid hydrazide or pyrene acetic acid hydrazide is attached to single-strand RNA 3' termini a red shift in absorbance and substantial hypochromicity are observed. A strong induced CD is seen and the fluorescence intensity is quenched by an order of magnitude. In double-stranded samples, a further 10-fold quenching of fluorescence is seen. Several lines of evidence suggest that the residual fluorescence of pyrene butyric acid hydrazide-duplex conjugates arises from a minor species. The most likely possibility is dye reacted at a site other than the 3' end. Some indication exists that 3'-attached pyrene may perturb the relative stability of nearby duplex. Within the limits of this reservation, it appears that 3'-pyrene conjugates may be rather useful for detecting the existence of duplex regions accessible to a dye at the 3' end of complex RNAs.

INTRODUCTION

There has been considerable interest in the use of pyrene derivatives as fluorescent probes of proteins or nucleic acids. The unusually long singlet lifetime of pyrene is potentially a great advantage for fluorescence anisotropy measurements on large systems, for dynamic quenching studies, and for accurate energy-transfer measurements. Great experimental sensitivity is afforded by the large extinction coefficient and high quantum yield of pyrene and some derivatives. These advantages are sometimes offset by the tendency of pyrene to form excimers,¹ its poor solubility in aqueous systems, and occasional observations of apparent nonexponential fluorescence decay.²

In the past the pyrene fluorophore has been used directly through non-covalent binding³ and as a covalent probe of proteins by the use of pyrene butyric acid anhydrides⁴ and a pyrene maleimide.⁵ Some time ago we reported the preparation of pyrene butyric acid hydrazide (PBH), a probe capable of reacting with aldehydes generated by periodate oxidation of RNA 3' termini.⁶ Here the properties of a number of PBH-3'-RNA conjugates will be shown. The preparation of a related probe, pyrene acetic acid hydrazide (PAH), will also be described.

MATERIALS AND METHODS

Synthesis of PAH

Pyrene acetic acid was synthesized using a modification of the method of Bachman and Carmack.⁷ 6.6 g of AlCl_3 were added to 20 ml of nitrobenzene in a flask in an ice-salt bath (-5°C). To this, 2.6 ml of acetic anhydride were added, and then 5.0 g of finely ground pyrene were slowly added with good stirring. The temperature was raised to 10°C and stirring continued for 7 hr, the last 4 hr at 0°C . Ice was added with shaking, then 20 ml conc HCl were added and the sample was stirred overnight. The mixture was extracted with ether and this extract was evaporated to dryness. The yellow 1-acetyl pyrene was recrystallized from methanol and dried under vacuum.

The Friedel-Crafts acetyl product was converted to an amide by the Willgerodt reaction. 1 g of sulfur powder was added to 10 ml of concentrated aqueous NH_3 and then H_2S was bubbled through the mixture until the sulfur dissolved. This solution was then added to a glass bomb along with 8 ml dioxane and 2 g of 1-acetyl pyrene. The sealed bomb was heated at $165\text{--}180^\circ\text{C}$ for 12 hr, then cooled, and the contents removed and filtered to collect the brown 1-pyrene acetamide crystals.

The amide (0.826 g) was dissolved in 12 ml of glacial acetic acid and refluxed. Then 6 ml of concentrated HCl were slowly added through the condenser. After 75 min of reflux an additional 6 ml of HCl were added and a yellow precipitate formed. After chilling, the yellow crystals of 1-pyrene acetic acid were collected by filtration and recrystallized from chlorobenzene. The product was purified by silica gel thin-layer chromatography using either hexane/ethyl acetate (3:2) with several drops of acetic acid or ethyl acetate/methanol/ H_2O / NH_4OH (10:2:1:1).

To convert to the hydrazide, 0.10 g of the 1-pyrene acetic acid was added to 30 ml of ethanol along with 20 mg of *p*-toluene sulfonic acid. The mixture was refluxed and after the first and second hours 10 ml were distilled off and replaced by 10 ml of fresh ethanol. The reflux was continued overnight and then solvent was evaporated under vacuum. The product was put through a silica gel column with CHCl_3 , and fractions containing the ester were pooled and evaporated to yield a brown oil which slowly produced yellow crystals. The crude pyrene acetic acid ethyl ester (0.14 g) was added to 0.6 ml hydrazine hydrate and refluxed for 10 min. Then 8 ml of ethanol were added and the sample was refluxed overnight. The mixture was filtered, cooled, and the resulting yellow crystals of PAH were collected by filtration and dried under vacuum.

Sources of Homopolynucleotides, tRNA, 16S rRNA

Homopolymers were obtained commercially, poly (U) from Schwarz/Mann, poly (I) from Biopolymers, poly (A) and poly (C) from Miles, and were used without further purification. 16S rRNA was kindly provided by Dr. Nancy Hsiung. *E. coli* tRNA^{Met} was provided by Dr. A. D. Kelmers

of Oak Ridge National Laboratory. Unfractionated baker's yeast tRNA (tRNA_{yeast}^{UNF}) was obtained from Plenum Scientific Research, Inc. Purified tRNA^{Phe} from yeast was obtained from Boehringer-Manheim Biochemicals.

Preparation of Fluorescent Conjugates

In a typical labeling reaction 5 mg of polymer were dissolved in 1 ml of 6M urea, 0.05M acetate buffer (pH 5.6), along with 10 mg NaIO₄. The reaction mixture was kept in the dark at room temperature for 45 min. Then 0.1 ml of 2M KCl was added and the solution kept at 4°C for 1 hr, after which the KIO₄ crystals were removed by filtration, and the polymer precipitated by addition of 2 ml ethanol and cooling to -20°C. The polymer was then redissolved in 1 ml of the acetate buffer. To this was added 1 ml of dimethylsulfoxide (Me₂SO) containing 1 mg PBH. The solution was incubated for 2 hr at 37°C, and then the polymer was precipitated by adding 0.1 ml 2M NaCl and 2 ml ethanol. The precipitate was centrifuged out and redissolved in the buffer 0.01M Tris, 0.003M disodium EDTA, and 0.1M NaCl (abbreviated TeN), pH 7.5. The precipitation procedure was repeated at least five times until the supernatant no longer showed any fluorescence.

Determination of ϵ

The absorption coefficient of labeled polymer in TeN, pH 7.5, was determined indirectly. Labeled polymer in TeN, pH 7.5, was diluted 10-fold in the same buffer; another identical sample was diluted 10-fold with Me₂SO. The same dilutions were made on two identical samples of pyrene butyric acid (PBA) in 0.2M borate buffer (pH 8.9). The extinction of PBA in 90% Me₂SO, ϵ_{346} can be computed from the measured absorbances of the PBA samples using the known ϵ_{342} of PBA in buffer, 40,500,⁴ because both samples have identical concentrations.

$$\epsilon_{346}^{\text{Me}_2\text{SO}} = \frac{A_{346}^{\text{Me}_2\text{SO}}}{A_{342}^{\text{buffer}}} \epsilon_{342}^{\text{buffer}}$$

The ϵ_{max} of free PBA and PBH on polymers in 90% Me₂SO were assumed to be equal because Me₂SO is known to be an efficient disruptor of secondary structure.⁸ For example, λ_{max} in both cases has shifted to 346 nm. The assumption about the ϵ_{max} of bound PBH in Me₂SO is likely to be quite accurate, and, in any event, is unlikely to change significantly any of the results in this paper except for the actual quantitative hypochromicity values. Using this assumption, the extinction at λ_{max} of each polymer can be computed from the ratio of the absorbance of the two polymer samples using the fact that their concentrations are equal:

$$\epsilon_{\text{max}}^{\text{buffer}} = \frac{A_{\text{max}}^{\text{buffer}}}{A_{346}^{\text{Me}_2\text{SO}}} \epsilon_{346}^{\text{Me}_2\text{SO}}$$

Optical Measurements

Circular dichroism (CD) spectra were obtained on a Jasco circular dichroism spectrometer, model J-40, in water-jacketed 0.1-cm or 1.0-cm quartz cells at 25°C. Fluorescence spectra were measured on a Perkin-Elmer MFP 2A fluorimeter in 1-cm quartz cells. Fluorescence lifetimes were determined using a single photon counter described previously.⁹ Quantum yields were determined as described previously, using quinine sulfate in 0.1 NH_4SO_4 as a standard.⁶

Absorption spectra were run in matched 1-cm quartz cells in Cary 15 or 118 spectrometers. Titration curves were obtained by adding polymer in different molar ratios to a series of tubes, each containing the same amount of labeled polymer. The tubes were mixed and fluorescence measured after at least 10 min or after any time dependence had terminated.

All optical measurements were done on solutions in TeN, pH 7.5, unless otherwise noted. PBA was measured in 0.2M borate buffer, pH 8.9. For measurements between pH 4.0 and pH 5.6, a buffer of 0.05M NaOAc, 0.05M NaCl was used.

RESULTS

PBH and PAH were reacted with a variety of 3'-oxidized RNAs (Table I). The length of the synthetic polymers was not determined, but for the tRNA the extent of labeling was 0.83 dye molecules for each molecule of tRNA. In all cases the absorption spectrum of the bound conjugate shows a shift to the red and substantial hypochromicity relative to pyrene butyric acid. The magnitude of the hypochromicity of the different dye polymer conjugates is found to be directly related to the extent of the red shift of λ_{max} .

Both of these effects are probably due to stacking of the pyrene moiety with the nucleotide bases of the polymers, bringing the pyrene into a hydrophobic environment. The exact nature of the microenvironment is presumably determined by the nature of the polymer. Dimethylsulfoxide, a known disrupter of polynucleotide secondary structure,⁸ obliterates these differences (Fig. 1) and can be used to determine extinction coefficients of bound pyrene, as explained in "Materials and Methods."

Further evidence of the interaction between 3'-attached pyrene and the nucleotides is shown by circular dichroism studies (Table II and Fig. 2). PBA itself shows no CD. When the pyrene is conjugated to an RNA polymer, however, in virtually all cases optical activity in pyrene absorption bands is observed. The induced CD shown depends in a delicate way on the average orientation of pyrene and nearby nucleotides and the pattern which emerges is complex. The magnitudes of $[\theta_{\text{max}}]$ vary widely and do not seem to be correlated with the shift in wavelength of the major peak. Poly (A) of pH 7.5 was the only sample examined which failed to show an intense induced CD. However, both absorption and fluorescence data

TABLE I
 Absorbance of Pyrene Conjugates^a

	λ_{\max} (nm)	ϵ_{\max} ($\times 10^{-4}$)	Hypochromicity (%)	Pyrene/ Nucleotide ($M/M \times 10^4$)
PBA (pH 8.9)	342	4.05	—	—
PBH-poly (C)	342	3.7 ^b	8.9	7.30
PBH-poly (I)	343	3.14	22.7	16.2
PAH-poly (C)	344	3.2 ^b	21.2	8.98
PBA (benzene)	345	—	—	—
PBH-poly (U)	345	2.73	32.8	1.70
PBH-poly (A)	348	2.61	35.7	6.23
PBH-tRNA _{yeast} ^{UNF}	349	2.06	49.2	106

^a All solutions at pH 7.5 unless otherwise noted.

^b Interpolated values using the apparent monotonic decrease in ϵ_{\max} with increasing λ_{\max} .

indicate that in the sample pyrene is strongly interacting with nucleic acids. Presumably, the geometry of the complex formed is such that various contributions to the induced CD approximately cancel.

An indication of the sensitivity to precise geometric arrangement of dye and nucleotides is seen by comparing the spectral data of PAH and PBH

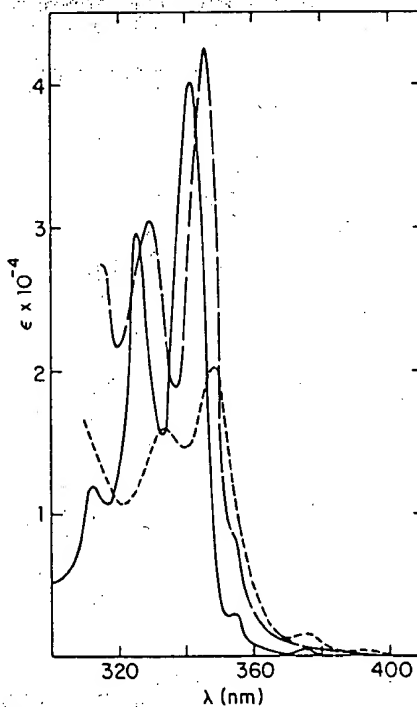


Fig. 1. Absorption spectra of free pyrene butyric acid and PBH-tRNA conjugates. — PBA in borate buffer pH 8.9; - - - PBH-tRNA_{yeast}^{UNF} in TeN, pH 7.5; — — PBH-tRNA_{yeast}^{UNF} in 90% Me₂SO and PBA in 90% Me₂SO (assuming that the molar extinction coefficients of both are the same).

TABLE II
Circular Dichroism of Pyrene Conjugates^a

	λ_{\max} (nm)	θ_{\max} ($\times 10^{-4}$) ^b
PBH-poly (U)	347	0.41
PBH-poly (C)	351	2.4
PAH-poly (C) (pH 8.2)	338	-0.68
PAH-poly (C) (pH 4.2)	353	1.1
PBH-poly (I)	345	0.79
PBH-poly (A)	—	0
PBH-poly (A) (pH 5.6)	354	-2.3
PAH-poly (A) (pH 4.2)	351	-2.5
PBH-tRNA ^{Met} _{E. coli}	350	0.64
PBH-tRNA ^{UNP} _{yeast}	349	0.64
PBH-tRNA ^{Phe} _{yeast}	348	0.50
PBH-16S RNA	348	0.48

^a All solutions at pH 7.5 unless otherwise noted.

^b Pyrene molar ellipticity computed spectrophotometrically using extinction coefficients in Table I or those of closely related species.

on both poly (A) and poly (C). With both polymers the change from a pyrene two-carbon to a pyrene four-carbon chain conjugate produces a large change in CD. Similarly, when the pH is lowered, converting the poly (C) and poly (A) conjugates from single- to double-stranded forms,^{10,11} a substantial change in the CD is observed. Furthermore, on the addition of

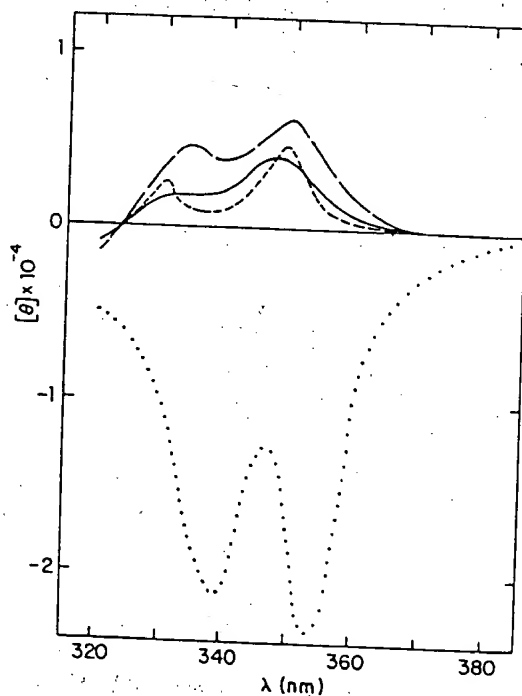


Fig. 2. Circular dichroism of pyrene butyric acid hydrazide conjugates. — PBH-tRNA^{UNF}_{yeast}, TeN pH 7.5; — PBH-poly (U), TeN pH 7.5; - - - - PBH-poly (A) + 2-fold excess of poly (U), 0.01M Tris, 0.03M EDTA, 0.1M NaCl, pH 7.4; PBH-Poly (A), 0.5M acetate, 0.5M NaCl, pH 5.6.

TABLE III
 Fluorescence of Pyrene Conjugates^a

	$\lambda_{\text{max}}^{\text{em}}$ (nm)	Quantum Yield
PBA (pH 8.9)	377	0.50
PBH-poly (C)	377	0.053
PBH-poly (I)	378	0.073
PBH-poly (U)	379	0.016
PBH-poly (A)	379	0.084
PBH-tRNA ^{UNF} _{yeast}	378	0.023
PBH-poly (A) (pH 4.5)	378	0.0049 ^b
PBH-poly (C) (pH 4.0)	378	0.0059 ^b
PBH-poly (A)-poly (U)	c	0.017 ^b
PBH-poly (U)-poly (A)	c	0.045 ^b
PBH-poly (I)-poly (C)	c	0.030 ^b
PBH-poly (C)-poly (I)	c	0.026 ^b

^a All solutions are pH 7.5 unless otherwise noted.

^b Relative values calculated from single-wavelength fluorescence changes.

^c Not determined.

Me₂SO (to destroy the secondary structure), the CD is abolished, showing that the CD is the result of specific interactions between dye and nucleotides and not merely the result of chemical conjugation.

Changes in fluorescence (Table III) are the most dramatic indication that pyrene attached to RNA 3' termini senses some aspects of local structure there. The pyrene fluorescence is quenched approximately tenfold on

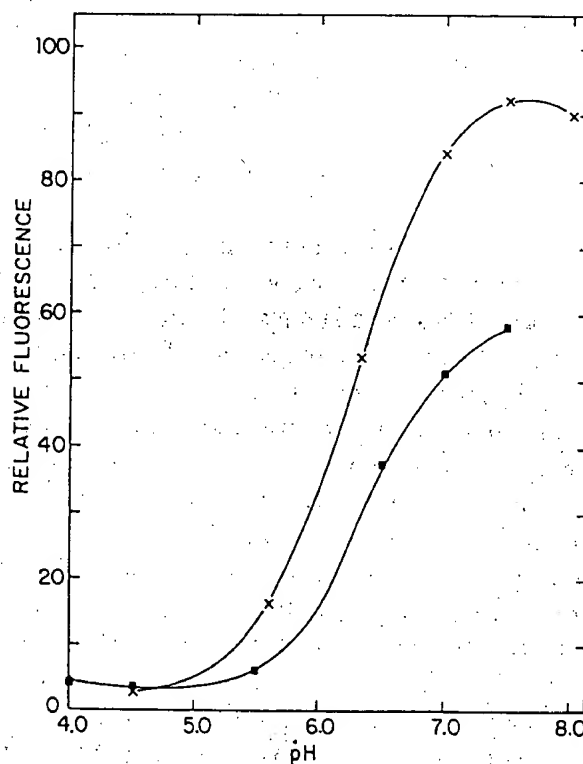


Fig. 3. Fluorescence of PBH-poly (A) (X) and PBH-poly (C) (■) as a function of pH.

conjugation to a single strand, and another tenfold when single strands are converted to double-stranded forms. Whatever the exact molecular mechanism, PBH is obviously a sensitive probe of secondary structure. For example, the pH-dependent single-strand to double-strand conversion of poly (A) and poly (C) was monitored by following PBH fluorescence (Fig. 3). The quenching on going from single- to double-stranded forms appears to result from a more efficient integration of the dye into the ordered double-stranded structure. Dimethylsulfoxide has a dramatic effect on the fluorescence of all double-stranded PBH-conjugates. By 50–60% Me_2SO the fluorescence is increased up to the levels seen in single strands under similar conditions.

By extrapolation one might speculate that if in double-stranded structures pyrene were inserted into a perfectly ordered double strand the fluorescence would be totally quenched. The small amount of fluorescence remaining in the double-stranded structures might then represent the fluorescence of a minor species. This could be due to some polymer molecules with 3' termini not base paired, occasional pyrenes attached at places other than the 3' end or simple equilibrium between intercalated dyes and those in contact with solvent.

The idea that the pyrene fluorescence in double strands comes from only a subset of the attached probes is supported by evidence that the probe population which yields the absorption spectrum is not identical to the fluorescing population. Corrected excitation spectra do not match corresponding absorption spectra, but resemble rather the absorption of free PBA (Table IV). The fluorescence lifetimes of PBH conjugates are not shortened to the same extent as the quantum yields. One component of the conjugate lifetime spectrum is nearly as long as the lifetime of free PBA (Fig. 4). In addition, I^- quenching experiments show that the fluorescent species is quenched with a collisional rate constant similar to that of free PBA (Table V), implying that the fluorescing species is at least partially exposed.

These results suggest that the residual pyrene fluorescence in double-stranded samples is due to a minor species, but they do not distinguish among the three possibilities raised above. Indirect evidence tends to favor the idea of occasional pyrene side reactions. There is precedent for the direct reaction of hydrazines with nucleic acid bases.¹² Indeed, RNA samples which have not been periodate oxidized typically react covalent with PBH at about 1% the level of oxidized samples.⁶ The apparent relative quantum yields of pyrene in double-stranded structures are always less than single strands, but absolute magnitudes are not reproducible. This could be explained by variability in the extent of such nonspecific hydrazine reaction. Because of the almost total quenching of 3'-attached PBH even 1% side reaction would be sufficient to explain the residual fluorescence in double strands. This implies that when pyrene fluorescence is used to monitor conformation changes the most accurate procedure may be to subtract any observed duplex fluorescence and use only the remainder as an indicator of the fraction of molecules with unpaired ends.

TABLE IV
Corrected Excitation of Pyrene Conjugates

	Absorption λ_{\max} (nm)	Excitation λ_{\max} (nm)
PBA (0.2M borate, pH 8.9)	342	342
PBA (90% Me ₂ SO)	346	346
PBH-tRNA ^{UNF} (90% Me ₂ SO)	346	346
PBH-tRNA ^{UNF} (TeN, pH 7.5)	348	343
PBA (benzene)	345	344

A large fluorescent probe like PBH has the inherent risk of inducing significant structural perturbations. Usually it is difficult to obtain direct evidence for or against this possibility. In the case of homopolymer duplexes, however, we have seen some indications that 3'-attached PBH alters the relative stability of duplex regions near the site of dye attachment. When PBH is attached to poly (A) or poly (I), subsequent addition of poly (U) or poly (C), respectively, yields fluorescence changes which parallel a normal titration (Fig. 5). However, when PBH labeled poly (C) is titrated with poly (I), the fluorescence indicates completion of duplex formation at a stoichiometry significantly less than 1 poly (I) = 1 poly (C). Presumably poly (I) prefers to bind to 3' ends of the poly (C) in the vicinity of the dye.

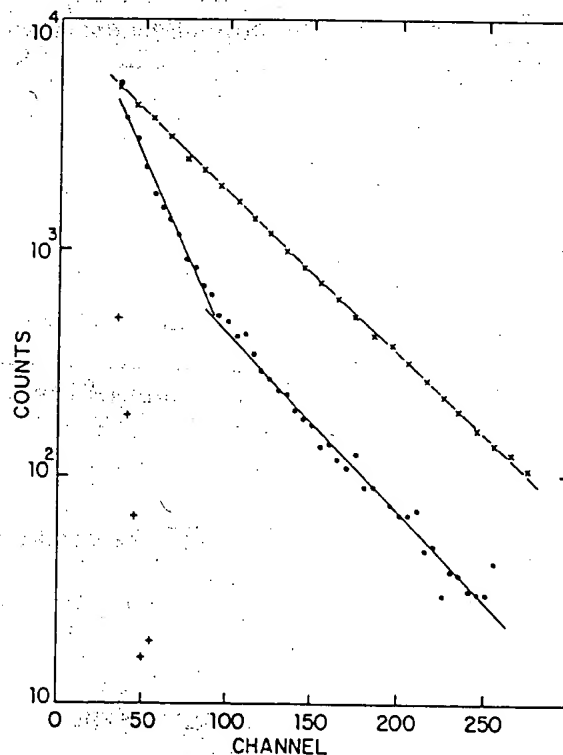


Fig. 4. Fluorescence decay of free PBA in borate buffer, pH 8.9 (x) and PBH-tRNA in 0.01M Tris, 0.01M MgOAc, pH 7.5 (-). The tail of the exciting pulse is also indicated (+). Straight lines through sections of the decay correspond to lifetimes of 45 and 82 nsec for PBH-tRNA and 94 nsec for PBA. One channel is equal to 1.55 nsec.

TABLE V
Iodide Quenching of Pyrene and Pyrene Conjugates^a

	PBA (pH 8.9)	PBH-tRNA _{yeast} ^{UNF}	PBH-Poly (U)
τ_0 (sec)	118.6×10^{-9}	84.2×10^{-9}	106×10^{-9}
K_q (L/mol sec)	4.33×10^8	2.67×10^8	1.77×10^8

^a For collisional quenching $F_0/F = 1 + K_q \tau_0 [Q]$, where F_0 and τ_0 are the fluorescence and fluorescent lifetimes, respectively, in the absence of quencher. F is the fluorescence at a given concentration of quencher $[Q]$. K_q is the apparent collisional rate constant.

In contrast, titration of labeled poly (U) with poly (A) is surprisingly complicated, as shown in Fig. 5. The low level of fluorescence of PBH-poly (U) in the absence of poly (A) suggests an ordered structure. One possible explanation is that PBH induces a poly (U)-poly (U) duplex structure¹³

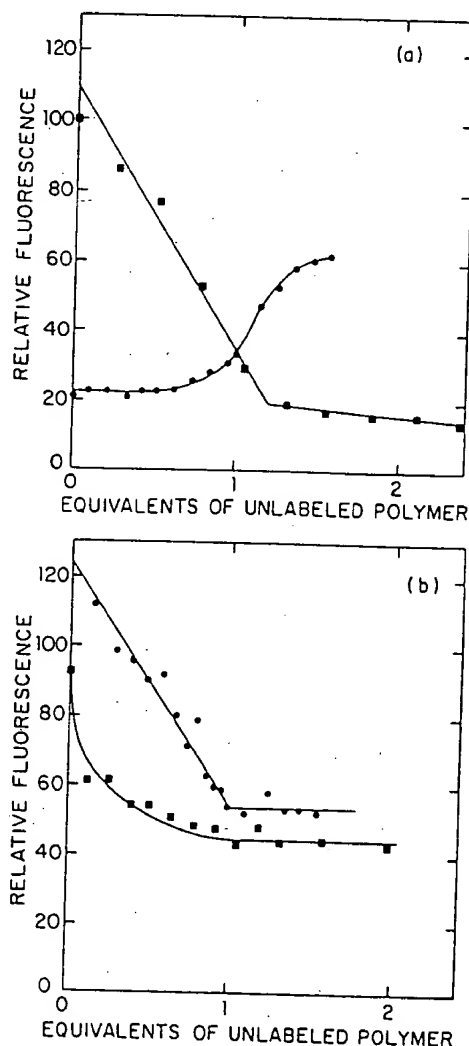


Fig. 5. Effect of complementary polynucleotides on the fluorescence of PBH-conjugates. In each case the fluorescent species was held at constant concentration and increasing amounts of complementary polymer were added successively. (a) ●, poly (A) added to PBH-poly (U); ■, poly (U) added to PBH-poly (A); (b) ●, poly (C) added to PBH-poly (I); ■, poly (I) added to PBH-poly (C).

near its site of attachment. There is appreciable hypochromism and CD induced in PBH bound to poly (U) in spite of the fact that this polymer is believed to have little or no base stacking at room temperature in dilute salt.¹⁴ This may reflect a pyrene-induced formation of stacked or duplex structures. Further evidence comes from the marked temperature dependence of the CD of PBH-poly (U). $[\theta_{\max}]$ is halved when the temperature is raised from 2 to 27°C. Under the conditions used for the experiment shown in Fig. 5(a), poly (A)-poly (U) mixtures will form triple-strand poly (A):2 poly (U) as long as the poly (U):poly (A) ratio is greater than 1:1.¹⁵ Apparently this proceeds without significant change in the fluorescence of PBH at the 3' end of poly (U). As more poly (A) is added, the 2:1 complex will convert to poly (A)-poly (U). This is accompanied by an increase in pyrene fluorescence. It appears that 3'-PBH poly (U) derivatives may be very useful in discriminating between triple- and double-stranded poly (U)-containing structures.

DISCUSSION

The results presented above indicate that 3'-attached PBH or PAH can detect the presence of nearby duplex structure in polynucleotide complexes. A Watson-Crick duplex is not required, since acid double-helices of poly (A) and poly (C) show similar quenching. It is not necessary that the actual residue to which the PBH is attached be involved in duplex. PBH-3'-tRNA conjugates show a comparable quenching. There is every indication in the crystal^{16,17} and in solution^{18,19} that the 3' CCA end is not base-paired in tRNA. Our suspicion is that PBH attached at the 3' end can bend back and intercalate into the adjacent stem duplex of the tRNA. Since PAH and PBH show similar effects, there is apparently some flexibility in the approach of the dye to the site which causes its quenching. Since PBH-3'-16S rRNA conjugates show the characteristic duplex quenching, there must be a double-strand region accessible to a dye on the 3' end. This may be the hairpin loop suggested to exist near the 3' end^{20,21} or it may be any nearby double-stranded region.

It appears that PBH and PAH conjugates may be useful for exploring conformation near the 3' ends of various RNAs. The large fluorescence changes and great intensity of the free chromophore encourages experiments on viral or mRNAs and fragments which may be difficult to prepare in sufficient quantities for more conventional techniques.

The authors are grateful to Mr. R. H. Fairclough and Drs. R. G. Langlois, and P. Solomon for their helpful comments.

This work was supported by a grant, GM14825, from the USPMS. Scott Reines was an NIH postdoctoral fellow, GM 49534-03.

References

1. Parker, C. A. (1968) *Photoluminescence of Solutions*, Elsevier, New York, pp. 363-366.
2. Parker, C. A. & Hatchard, C. G. (1963) *Trans. Faraday Soc.* 59, 284-295.

3. Luk, C. K. (1971) *Biopolymers* 10, 1317-1329.
4. Knopp, J. A. & Weber, G. (1969) *J. Biol. Chem.* 244 (23), 6309-6315.
5. Weltman, J., Szaro, R. P., Frackelton, Jr., A. R., Dowben, R. M., Bunting, J. R. & Cathou, R. E. (1973) *J. Biol. Chem.* 248, 3173-3177.
6. Reines, S. & Cantor, C. R. (1974) *Nucleic Acids Res.* 1, 767-786.
7. Bachman, W. E. & Carmack, M. (1941) *J. Am. Chem. Soc.* 63, 2494-2499.
8. Strauss, Jr., J. H., Kelly, R. B. & Sinsheimer, R. L. (1968) *Biopolymers* 6, 793-807.
9. Tao, T. (1969) *Biopolymers* 8, 609-632.
10. Holcomb, D. N. & Tinoco, Jr., I. (1965) *Biopolymers* 3, 121-133.
11. Hartman, Jr., K. A. & Rich, A. (1965) *J. Am. Chem. Soc.* 87, 2033-2039.
12. Hall, R. H. (1971) *The Modified Nucleotides in Nucleic Acids*, Columbia University Press, New York, pp. 356-358.
13. Thrierr, J. C., Dourlent, M. & Leng, M. (1971) *J. Mol. Biol.* 58, 815-830.
14. Simpkins, H. & Richards, E. G. (1967) *Biopolymers* 5, 551-560.
15. Krakauer, H. & Sturtevant, J. M. (1968) *Biopolymers* 6, 491-512.
16. Kim, S. H., Suddath, F. L., Quigley, G. J., McPherson, A., Sussman J. L., Wang, A. H. J., Seeman, N. C. & Rich, A. (1974) *Science* 185, 435-440.
17. Robertus, J. D., Ladner, J. E., Finch, J. T., Rhodes, D., Brown, R. S., Clark, B. F. C. & Klug, A. (1974) *Nature* 250, 546-551.
18. Uhlenbeck, O. C. (1972) *J. Mol. Biol.* 65, 25-41.
19. Gamble, R. C. & Schimmel, P. R. (1974) *Proc. Natl. Acad. Sci. U.S.* 71, 1356-1360.
20. Dahlberg, A. E. & Dahlberg, J. E. (1975) *Proc. Natl. Acad. Sci. U.S.* 72, 2940-2944.
21. Ehresmann, C., Stiegler, P., Mackie, G. A., Zimmermann, R. A., Ebel, J. P. & Fellner, P. (1975) *Nucleic Acids Res.* 2, 265-278.

Received September 13, 1976

Accepted February 21, 1977

On the Phe-tRNA induced binding of fluorescent oligonucleotides to the ribosomal decoding site

Heinrich M. Menzel

Fachgebiet Biochemie, Technische Hochschule Darmstadt, Petersenstr. 22, D-6100 Darmstadt, GFR

Received 9 June 1977

ABSTRACT

Fluorescent oligonucleotides were prepared by dansylation of 5'-amino uridylates of varying chainlength. Except for the trinucleoside diphosphate, they stimulated the binding of Phe-tRNA to 70S E. coli ribosomes as efficiently as underivatized oligouridylic acids of comparable chainlength. The ternary ribosomal complex $[70S \times \text{Phe-tRNA} \times \text{dansyl-n}^5\text{U(pu)}_4]$ was separated from excess oligonucleotide and its fluorescence spectra were measured. The quantum yield of the dansylated pentauridylylate was enhanced 2.5 fold when bound to the ribosomal decoding site, but no shift of the emission spectrum was observed. The ribosomal complex is considered useful for topographic investigations by singlet energy transfer, using the functionally defined decoding site as reference point.

INTRODUCTION

Due to its sensitivity and versatility fluorescence techniques have been successfully used to investigate the structure of ribosomes (1 - 11). Random labelling with fluorescent probes yields heterogeneous populations of ribosomes, which permit the determination of overall properties (1 - 5). By use of isolated ribosomal proteins, which were labelled at random, the kinetics of 30S reconstitution (9) was studied and protein neighbours were determined (10, 11). The latter results were obtained by singlet energy transfer experiments, assuming the corresponding proteins to be globular in the ribosomal RNA-protein complex, which does not hold for all proteins (12). Nevertheless, the results are in good agreement with those obtained by other techniques.

In contrast to randomly labelled ribosomes, the introduction of fluorescent probes at distinct, functionally defined sites has the advantage of making the interpretation of the results less ambiguous. This goal has been achieved by Cantor et

al. (13) with the aid of fluorescent antibiotics, which permitted the determination of the distance between the erythromycin binding site and the proteins L7, L12 (14). Wintermeyer et al. (15) were able to form ribosomal complexes with a macromolecular ligand, tRNA^{Phe} and Phe-tRNA^{Phe} from yeast, which was specifically derivatised with ethidium bromide in the dihydrouridine loop or the anticodon loop. Despite of these successes, alternative ways for the specific labelling of ribosomes are warranted, because thereby the possibilities for both topographic and functional studies are enlarged.

Encouraged by the finding, that derivatised oligonucleotides retain their ability to function as messenger analogues (16, 17), I considered the specific introduction of a fluorescent label into the ribosomal decoding site as feasible. The present paper demonstrates, that this is possible and that the use of dansylated oligonucleotides has two advantages: Due to the separation of products, quantitatively labelled messenger analogues of high purity can be synthesised, and their binding to ribosomes is made specific by the interaction with the cognate aminoacyl-tRNA.

MATERIALS AND METHODS

Preparation of dansyl-n⁵'U(pu)_x:

Introduction of the dansyl group was always the last step, starting from the corresponding 5'-amino uridylates, which were prepared by standard procedures (18 - 21). Usually 0.5 μmol of the potassium salt of n⁵'U(pu)_x was dissolved in 2 ml 50 mM sodium bicarbonate buffer (pH 9.2), which contained 50% (v/v) acetone and 50 μmol of 1-dimethylamino-5-naphtalenesulfonyl chloride (Aldrich Europe). After stirring for 9 hours at room temperature in the dark, the clear solution was extracted with ethyl acetate and dansylic acid was precipitated with acetic acid. The supernatant was diluted with 20 ml 0.1 M triethyl ammonium bicarbonate (pH 7.4) and chromatographed on a DEAE cellulose column (1.5 cm x 6 cm), which was developed with a linear triethyl ammonium bicarbonate gradient (250 ml 0.0 M to 250 ml 0.4 M). The reaction products chromatographed well ahead of the starting oligonucleotides and were separated from fluorescent impurities. Homogeneity and composition of dansyl-n⁵'U(pu)_x was

determined by paper electrophoresis, pH 2.5 and pH 7.5, paper chromatography in n-butanol:acetic acid:water 5:2:3, and by nucleoside analysis (22). The products used for fluorescence measurements were free of fluorescent impurities.

70S ribosomes tight couples, Formation of Complexes:

Phase E. coli MRE 600 (E. Merck, Darmstadt) according to Noll (23). Crude tRNA from E. coli (Boehringer, Mannheim) was charged with [³H]phenylalanine (Amersham Buchler), specific activity 10³ Ci/mol, according to Traub (24), the degree of charging was 2%. The stimulation of Phe-tRNA binding to 70S ribosomes was determined in absence of supernatant factors as a function of oligonucleotide concentration. The incubation mixture contained in 0.1 ml 39 pmol 70S ribosomes, 44 pmol [³H]Phe-tRNA, and varying amounts of oligonucleotides. The salt concentration was identical to that in buffer A: 50 mM Tris-HCl (pH 7.4), 20 mM MgCl₂, 150 mM NH₄Cl. The incubation time was 10 minutes at 37°, followed by chilling in ice and millipore filtration according to Nirenberg and Leder (25). Blank values were determined in absence of oligonucleotide and amounted to 20% of the optimal value found at high messenger concentration. They were subtracted in each case. Formation of the ternary ribosomal complex was done by incubation of 1.4 nmol 70S ribosomes with 1.3 nmol [³H]Phe-tRNA and 75 nmol dansyl n⁵'U(pu)₄ in 0.15 ml buffer A (fig. 2a) under the conditions described above. In the control experiment Phe-tRNA was omitted (fig. 2b). Gel filtration was performed by use of a P-60 (Bio-Rad, München) column (1 cm x 30 cm) at 4° with buffer A as eluant. The arrow in fig. 2a indicated the fraction which was used for the kinetic and spectroscopic experiments. It contained 0.46 μM 70S ribosomes and 0.09 μM [³H]Phe-tRNA bound to ribosomes immediately after its collection. This was 30 minutes after the termination of the incubation at 37°. The kinetics of the ternary ribosomal complex dissociation (fig. 3) was analysed by plotting ln Y as function of time, where $Y = (F_t - F_\infty) / (F_0 - F_\infty)$. F is the relative intensity of emission at 520 nm or CPM found after millipore filtration, while subscripts refer to time. For F_∞ the value after EDTA addition was used.

Recording of spectra:

Absorption spectra of dansyl-n^{5'}U(pu)_x in buffer A were recorded at room temperature by use of a Cary 118C spectrophotometer. Excitation and emission spectra were obtained under the same conditions using a Perkin Elmer MPF-3 fluorescence spectrophotometer operating in the ratio mode. Slit settings were at 10 nm and the emitted light was filtered through a F 39 filter. Fluorescence spectra of the ribosomal complex were obtained by use of a Schoeffel RRS 1000 fluorescence spectrophotometer. Slit settings were at 6 nm for excitation and 15 nm for emission. The cuvette holder was thermostated at 0° and flushed with dry air. Emitted light was filtered through a GG 435 filter, because otherwise the solutions containing 0.5 μM 70S ribosomes showed very high fluorescence background with maxima at 558, 590 and 635 nm when excited at 340 nm. With the filter inserted, the background was small, but was corrected for by use of an equally concentrated solution of 70S ribosomes. The optical density at 340 nm, the excitation wavelength, was below 0.05 in case of the ribosomal complex and much lower in case of free oligonucleotides. No correction for inner filter effects was made due to the short pathlength (5 mm) of the rectangular cuvettes (Hellma GMBH, Mühlheim/Baden). If not otherwise mentioned, the excitation wavelength was always 340 nm and the emission wavelength 520 nm.

RESULTSCoding properties of dansyl-n^{5'}U(pu)_x:

Stimulation of Phe-trRNA binding to 70S E. coli ribosomes in absence of factor EF-T_u was determined as function of dansyl-n^{5'}U(pu)_x concentration. As shown in figure 1, no stimulation of Phe-trRNA binding is observed, when x was equal to two. For x greater than two, the curves are indistinguishable from that obtained for U(pu)₃, which was used as a reference in each binding assay. The same results were found when the stimulation of Phe-trRNA to 30S ribosomal subunits was investigated at 0° (data not shown).

Due to the high excess of oligonucleotide needed to induce Phe-trRNA binding, the isolation of the ribosomal complex is nec-

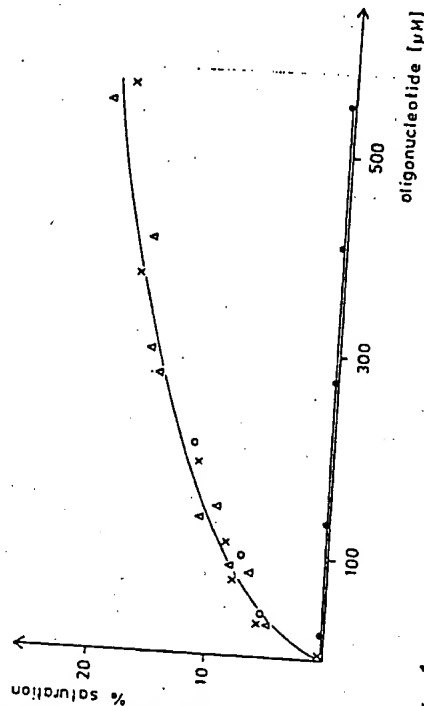


Fig. 1 Stimulation of the Phe-trRNA binding to 70S ribosomes by (X) U(pu)₃, (●) dansyl-n^{5'}U(pu)₂, (Δ) dansyl-n^{5'}U(pu)₄

essary in order to investigate its spectral properties. Figure 2a shows, that this can be achieved by gel filtration over a P-60 column. Omission of Phe-trRNA in the incubation mixture leads to the loss of dansyl-n^{5'}U(pu)₄ in the fractions containing the ribosomes (fig. 2b). The same finding was made when tritium labelled hexauridylylate was used as messenger analogue in the presence and absence of Phe-trRNA (data not shown).

In order to determine, whether the dissociation of [³H]Phe-trRNA and dansyl-n^{5'}U(pu)₄ were correlated, the time dependence of the complex stability was followed by radioactivity and fluorescence intensity. For this purpose, an aliquot of the incubation mixture was diluted 1:300 with buffer A, and kept under the same conditions as the incubation mixture during gel filtration and recording of the spectra. At appropriate times, the complex concentration was determined in this solution by millipore filtration (25) and by measurement of the relative emission intensity at 520 nm in the fraction 15 (see figure 2a). A semilogarithmic plot, which was normalised to the value at 30 minutes, yields the same straight line for the dissociation of Phe-trRNA and dansyl-n^{5'}U(pu)₄ from the ribosomal complex (fig. 3). The value chosen for infinite time was that obtained after

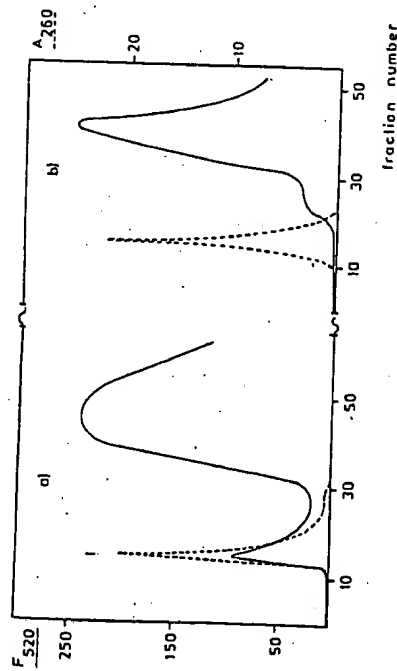


Fig. 2 Elution profile during gel filtration of 70S ribosomes and dansyl-n^{5'} U(pu)₄ in presence (fig.2a) and absence (fig.2b) of Phe-tRNA. A₂₆₀ (---) is absorbance and F₅₂₀ (—) is intensity of emission (in arbitrary units) at the respective wavelength

addition of EDTA in equal concentration to Mg²⁺. This treatment was observed to dissociate Phe-tRNA from ribosomes within less than three minutes at 0°. As shown in figure 3, the dissociation

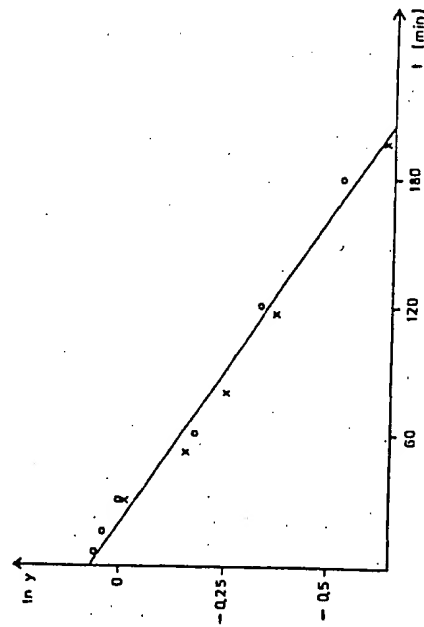


Fig. 3 Time course of the ribosomal complex dissociation. (X) refers to the intensity of emission at 520 nm and (O) refers to radioactivity of bound [³H]Phe-tRNA

tion of the ternary ribosomal complex is slow at 0°, with a half life time of about 210 minutes, and follows first order kinetics with respect to both Phe-tRNA and dansyl-n^{5'} U(pu)₄ dissociation.

Spectral properties of dansyl-n^{5'} U(pu)₄ and the ternary ribosomal complex:

Excitation and emission spectra of dansyl-n^{5'} U(pu)₄ at room temperature are shown in figure 4 as a function of the solvent. In aqueous solution, buffer A, the excitation spectrum exhibits a broad maximum around 335 nm, which does not exactly match the absorption spectrum, which has a shoulder at 330 nm. The extinction coefficient in 0.1 M sodium phosphate buffer (pH 7) at room temperature was determined to $\epsilon_{330} = 6 \times 10^3$ cm²mol⁻¹ by use of tritium labelled oligonucleotide. This value is somewhat higher than that found for comparable dansyl derivatives (26). Upon transfer of dansyl-n^{5'} U(pu)₄ from aqueous to methanolic solvent, the relative fluorescence intensity increases considerably (fig. 4) and the emission spectrum is shifted to shorter wavelengths (fig. 6). No change of the emission spectrum was observed for dansyl-n^{5'} U(pu)₄ at 0° in buffer A upon variation of the Mg²⁺ concentration between 0 and

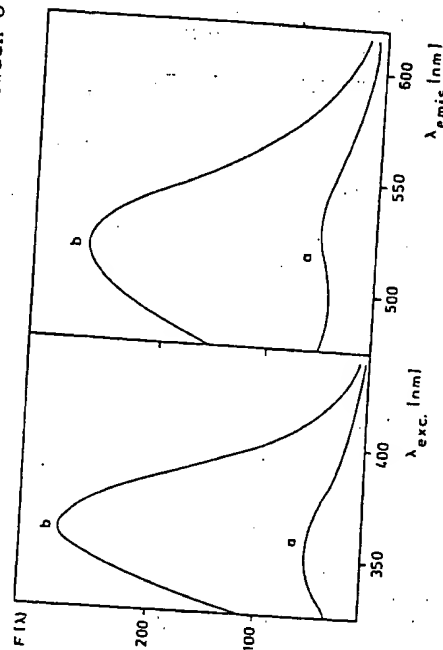


Fig. 4 Corrected excitation spectra and uncorrected emission spectra of 1.2 μM dansyl-n^{5'} U(pu)₄ in buffer A (a) and methanol (b) at room temperature. For details see materials and methods

20 mM, or upon addition of EDTA (data not shown). Furthermore, the fluorescence intensity at 440, 480 and 520 nm of the oligonucleotide in buffer A decreases linearly with increasing temperature between 0° and 32° by about 1% per centigrade (data not shown).

Uncorrected emission spectra of the isolated complex are given in figure 5. The fluorescence intensity decreases slowly with time at all wavelengths (see fig. 3) without shifting the emission spectrum. In contrast to free dansyl-n⁵'U(pU)₄, addition of EDTA in equal amounts to Mg²⁺, leads to an instantaneous drop of the emission intensity and then remains constant with time. In order to determine whether the dissociation of dansyl-n⁵'U(pU)₄ from the ribosome by EDTA shifts the emission spectrum, the ratio of relative intensities at each wavelength before (fig. 5a) and after (fig. 5d) dissociation was plotted as a function of wavelength (fig. 6). This procedure is preferred to the comparison of corrected emission spectra, because it is more sensitive to small changes and completely independent from instrumental parameters. Figure 6 shows, that upon dissociation of the ternary ribosomal complex by EDTA, the relative

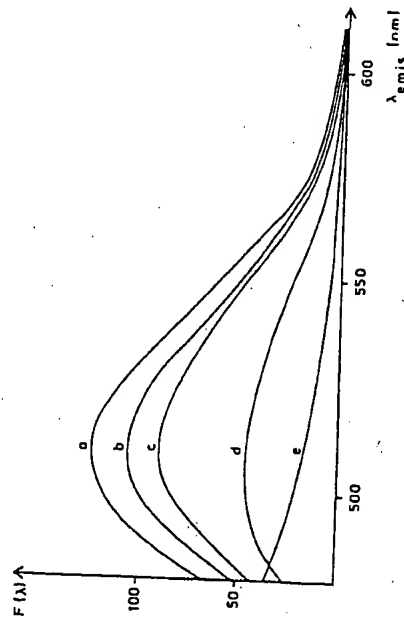


Fig. 5 Uncorrected emission spectra of the isolated ribosomal complex at 00 after (a) 30 minutes, (b) 80 minutes, (c) 200 minutes, and (d) after addition of EDTA. The lower curve (e) is the background due to ribosomes, for which all spectra are corrected

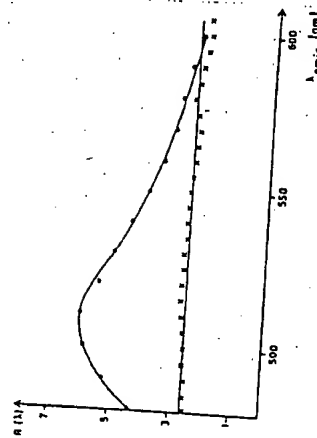


Fig. 6 Ratio of relative emission intensities of (●) a 1.2 μ M solution of dansyl-n⁵'U(pU)₄ in methanol/buffer A at room temperature and of (X) (ribosomal complex)/EDTA dissociated complex at 00

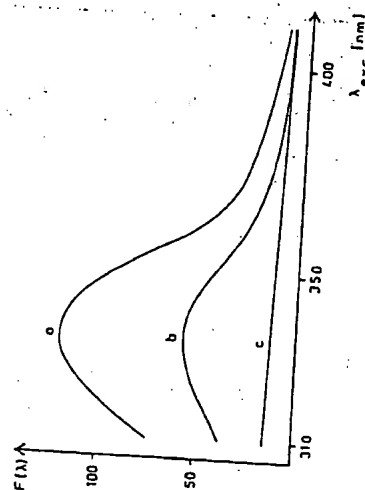


Fig. 7 Corrected excitation spectra of (a) the ribosomal complex (as in fig. 5a) and (b) the EDTA dissociated complex (as in fig. 5d) at 00 in buffer A. The spectra are corrected for the background (c) due to ribosomes

fluorescence intensity drops by a factor of 2.5, which is virtually independent from the wavelength.

The corrected excitation spectra of the ternary ribosomal complex before and after dissociation by EDTA are given in figure 7. As was found for the emission spectrum, the decrease in fluorescence intensity upon dissociation is independent from the excitation wavelength. This indicates, that no shift of the absorption spectrum of the dansyl moiety takes place when the oligonucleotide is bound to the ribosomal decoding site.

DISCUSSION

The assumption, that the isolated ribosomal complex contains the fluorescent oligonucleotide bound to the ribosomal decoding site, rests on the following findings: (a) dansyl-n⁵' U (pu)₃ fully substitutes U (pu)₃ as a messenger analogue in the EF-T_u independent binding of Phe-tRNA to 70S and 30S ribosomes, if the chainlength x is greater than two. The observed stimulation of Phe-tRNA binding cannot be explained by contaminating underivatized oligonucleotides, which were removed. If they were present in undetectable amounts and responsible for Phe-tRNA binding, the binding curves had to be shifted to much higher oligonucleotide concentrations, which is not the case. (b) If the cognate macromolecular ligand Phe-tRNA is omitted in the incubation mixture, no binding of dansyl-n⁵' U (pu)₄ to ribosomes is observed. This indicates, that unspecific binding is absent or much weaker than binding to the decoding site in presence of Phe-tRNA. (c) The time dependence of Phe-tRNA and dansyl-n⁵' U (pu)₄ dissociation from the complex is identical, which is in perfect agreement with the assumption, that dissociation is an all or none process for both aminoacyl-tRNA and messenger analogue. (d) The fast dissociation of both Phe-tRNA and dansyl-n⁵' U (pu)₄ from the complex by EDTA addition also confirms the interrelationship between the binding of the two ligands. Although none of this evidence by itself can be taken as proof, together it strongly indicates, that the ribosomal complex, formed according to the given procedure, contains the fluorescent oligonucleotide bound to the decoding site.

The lack of codon activity found for dansyl-n⁵' U (pu)₂ permits some assumptions to be made about the mode of dansyl-n⁵' U (pu)_x binding to the decoding site. While little difference exists between U (pu)₂ and U (pu)₃ with respect to the stimulation of Phe-tRNA binding, the observed difference in case of the dansylated uridylates suggests, that here the 5' terminal uridine is unavailable for base pairing with the anticodon loop. In the higher homologues, the dansyl residue therefore should be separated from this loop by at least one uridine base. Although halfsaturation points and plateau values determined for oligonucleotide induced binding of Phe-tRNA do not yield molecular parameters, the identity of the binding curves for

U (pu)₃ and dansyl-n⁵' U (pu)_x (with x greater than two) suggests, that these parameters are identical as well.

The solvent dependence of the quantum yield and the fluorescence emission spectrum of 1-dimethylamino-5-naphthalene-sulfonyl derivatives has been reported (26, 27) and were accounted for by a two state model (28). For dansyl-tryptophane Chen (27) reported a sixteenfold increase of fluorescence emission intensity at 510 nm and a pronounced blueshift of the emission spectrum, when the solvent was changed from water to methanol. Qualitatively the same finding is made for free dansyl-n⁵' U (pu)₄ (fig. 6), although the differences are not as pronounced as for dansyl-tryptophane. On dissociation of dansyl-n⁵' U (pu)₄ from the ribosomal decoding site, the quantum yield is much less affected and no blueshift of the emission spectrum is observed. From this it can be concluded, that the polarity in the close vicinity of the decoding site is higher than that of methanol and very close to that of water.

The formation of the ternary ribosomal complex [70S x dansyl-n⁵' U (pu)₄ x Phe-tRNA] offers an alternative route to introduce a fluorescent label specifically into the close vicinity of the ribosomal decoding site. Despite such drawbacks as lack of stability and the necessity to remove excess oligonucleotide, the complex is considered useful for the investigation of elementary steps of protein biosynthesis, such as translocation, and for topographic studies by singlet energy transfer. Furthermore, the small, but significant increase of quantum yield upon binding to the decoding site, makes investigation of the codon-ribosome interaction by fast kinetic techniques feasible.

ACKNOWLEDGEMENT

I am very grateful to professor H.G. Gassen for his interest and continuing support, which made this study possible. Furthermore, I am indebted to professor D. Riesner for the use of the Schoeffel fluorimeter at the Inst. Klin. Biochem. Physiol. Chem. Med. Hochsch. Hannover. This work was supported by a grant of the Deutsche Forschungsgemeinschaft.

REFERENCES

- 1 Cantor, C.R. (1971) *Trans. N.Y. Acad. Sci.* 33, 576-585
- 2 Barenboim, G.M., Domanski, A.N., and Turoverov, K.K. (1969) *Luminescence of Biopolymers and Cells*, Plenum Press, New York
- 3 Huang, K.H. and Cantor, C.R. (1973) *J. Mol. Biol.* 67, 265-275
- 4 Hsiung, N. and Cantor, C.R. (1973) *Arch. Biochem. Biophys.* 157, 125-132
- 5 Spitnik-Elson, P., Schechter, N., Abramovitz, R., and Elson, D. (1976) *Biochemistry* 15, 5246-5253
- 6 Pochon, F., Ekert, B., and Perrin, M. (1974) *Eur. J. Biochem.* 43, 115-125
- 7 Pochon, F. and Ekert, B. (1973) *Eur. J. Biochem.* 36, 311-316
- 8 Perrin, M. and Pochon, F. (1975) *Eur. J. Biochem.* 57, 319-324
- 9 Huang, K.-H. and Cantor, C.R. (1975) *J. Mol. Biol.* 97, 423-441
- 10 Huang, K.-H., Fairclough, R.H., and Cantor, C.R. (1975) *J. Mol. Biol.* 97, 443-470
- 11 Cantor, C.R., Huang, K.-H., and Fairclough, R.H. (1974) *Ribosomes (Meeting)* pp. 587-599, Nomura, M., Tissieres, A., and Lengyel, P. Eds., Cold Spring Harbor, New York
- 12 Lake, J.A., Pendergast, M., Kahan, L., and Nomura, M. (1974) *Proc. Natl. Acad. Sci. US* 71, 4688-4692
- 13 Hall, J., Davies, J.P., and Cantor, C.R. (1977) *Arch. Biochem. Biophys.* 179, 121-130
- 14 Janglois, R., Less, C.C., Cantor, C.R., Vince, R., and Pestka, S. (1976) *J. Mol. Biol.* 106, 297-313
- 15 Robertson, J.M., Kahan, M., Wintermeyer, W., and Zachau, H.G. (1977) *Eur. J. Biochem.* 72, 117-125
- 16 Lührmann, R., Gassen, H.G., and Stöffler, G. (1976) *Eur. J. Biochem.* 66, 1-9
- 17 Wagner, R. and Gassen, H.G. (1975) *Biochem. Biophys. Res. Commun.* 65, 519-529
- 18 Horwitz, J.P., Tomson, A.J., Urbanski, J.A., and Chua, J. (1962) *J. Org. Chem.* 27, 3045-3048
- 19 Rubinstein, M. and Patchornik, A. (1975) *Tetrahedron* 31, 2107-2110
- 20 Gassen, H.G. (1971) *FEBS-Lett.* 14, 225-229
- 21 Schettlers, H., Gassen, H.G., and Mathaei, H. (1972) *Biochem. Biophys. Acta* 272, 549-559
- 22 Gassen, H.G. and Leifer, W. (1970) *Z. Anal. Chem.* 252, 337-343
- 23 Noll, M., Hapke, B., Schreier, M.H., and Noll, H. (1973) *J. Mol. Biol.* 75, 281-294
- 24 Traub, B., Mizushima, S., Lowry, C.V., and Nomura, M. (1971) *Methods in Enzymology*, Vol. XX, pp. 391-408, Mollgave, K. and Grossmann, L. Eds. Academic Press, New York
- 25 Nirenberg, M. and Leder, P. (1964) *Science* 145, 1899-1907
- 26 Weber, G. (1952) *Biochem. J.* 51, 155-167
- 27 Chen, R.F. (1967) *Arch. Biochem. Biophys.* 120, 609-620
- 28 Li, Y.-H., Chan, L.-M., Tyler, L., Moody, R.T., Himel, C.M., and Hercules, D.M. (1975) *J. Am. Chem. Soc.* 97, 3118-3126

Photoreactivation and dark repair of ultraviolet light-induced pyrimidine dimers in chloroplast DNA

Gary D. Small and Carolyn S. Greimann

Division of Biochemistry, Physiology, and Pharmacology, Section on Biochemistry, The University of South Dakota, Vermillion, SD 57069, USA

Received 10 June 1977

ABSTRACT

A UV-specific endonuclease was used to detect ultraviolet light-induced pyrimidine dimers in chloroplast DNA of *Chlamydomonas reinhardtii* that was specifically labeled with tritiated thymidine. All of the dimers induced by 100 J/m² of 254 nm light are removed by photoreaction. Wild-type cells exposed to 50 J/m² of UV light removed over 80% of the dimers from chloroplast DNA after 24 h of incubation in growth medium in the dark. A UV-sensitive mutant, UVS 1, defective in the excision of pyrimidine dimers from nuclear DNA is capable of removing pyrimidine dimers from chloroplast DNA nearly as well as wild-type, suggesting that nuclear and chloroplast DNA dark-repair systems are under separate genetic control.

INTRODUCTION

The presence and biological importance of DNA in organelles of eukaryotic cells has been well documented¹. The existence in practically all cells of mechanisms for the repair of damaged DNA is also well established. Three DNA repair systems for coping with ultraviolet light-induced pyrimidine dimers have been elucidated primarily from work with bacteria; these are (1) photoreactivation, (2) excision-repair, and (3) postreplication repair. Clayton, Doda and Friedberg² failed to find evidence for any of these repair mechanisms operating on pyrimidine dimers induced in mitochondrial DNA of mammalian cells. Pyrimidine dimers can be removed from yeast mitochondrial DNA by photoreactivation although excision-repair is apparently absent^{3,4}.

The only previous study of chloroplast DNA repair failed to find evidence for excision of dimers⁵ or for repair replication⁶ in *Chlamydomonas* chloroplasts. However, these workers also reported a lack of excision of pyrimidine dimers from nuclear DNA of *Chlamydomonas*. Using a more sensitive assay for pyrimidine dimers, thus permitting a smaller fluence of irradiation, we found that *Chlamydomonas* does remove pyrimidine dimers from nuclear DNA in the dark⁷. We have extended the study of DNA repair in *Chlamydomonas* to chloroplast DNA. We find evidence for repair of pyrimidine dimers in chloroplast DNA both by photoreactivation and by a dark-repair process which may be excision-repair.

- 1-213.
 i. Commn.: 425-426.
 ryst. B29: 192-198.
 73. Acta Cryst. B29: 1641-1647.
 1-626.
 mmun. 37: 961-964.
 2.
 inication.
 Biochem. Biophys. Res. Commun. 52:
 macol. Rev. 12: 265-298.
 THERLAND. 1971. Cyclic AMP. Academic
 FURBERG. 1968. Science 159: 206-207.
 er. Chem. Soc. 94: 5070-5076.
 Nature [New Biol.] 244: 136-137; Acta
 published results.
 Proc. Nat. Acad. Sci. USA 71: 3325-3328.
 n Structure and Conformations of Nucleic
 s. Proc. IV Harry Steenbock Symp. M.
 d Conformations of Nucleic Acids and
 Larry Steenbock Symp. M. Sundaralingam
 Bioj. ers 13: 2061-2076.
 ADAMS & M. G. ROSSMANN. 1973. J. Mol.
 73. Biochemistry 12: 5101-5109.
 The Enzymes. D. D. Boyer, Ed.: 647-806.
 S. H. KIM. 1972. J. Mol. Biol. 66: 403-421.
 1972. Biochem. Biophys. Res. Commun. 48:

4E PAPER

f comment on the conformation of the
 are biologically active.
 side analogs that have been studied are
 changes are introduced at the carbohy-
 ive done there is change the stereocho-
 ie hydroxyl group from the "down"
 base. Conformation analysis of all the
 / diffraction show that they prefer the
 ve like normal nucleosides and nucleo-
 t the hydroxyl group up, you hinder
 vel, the C-2' endo hydroxyl group can
 i 5'-hydroxyl function. To what extent
 mpound to a given enzyme, we do not

FLUORESCENT NUCLEOSIDES AND NUCLEOTIDES*

Nelson J. Leonard and Glen L. Tolman
 Roger Adams Laboratory, School of Chemical Sciences
 University of Illinois, Urbana, Illinois 61801

INTRODUCTION

In investigating the three-dimensional structure of several biologically important macromolecules, various spectroscopic methods have been employed in gaining information concerning the correlation between molecular structure and biological function. In this regard, applications of fluorescence spectroscopy in the field of protein chemistry have been overwhelmingly successful, and many valuable structural and dynamic properties of macromolecular proteins have been obtained. Reviews outlining the many uses and advantages of fluorescence spectroscopy in the study of biologically important macromolecules are available.¹⁻⁴ Although similar experiments using fluorescence techniques in investigating the three-dimensional structure of nucleic acids are feasible, fluorescence spectroscopy has had limited use in nucleic acid chemistry because of the infrequent occurrence in nature of fluorescent nucleosides.⁴ Because of the attractive advantages that fluorescence techniques afford in studying the tertiary structure of molecules, we have endeavored to develop chemical means by which fluorescent probes can be introduced selectively into transfer RNA and into the dinucleotide coenzymes such as NAD⁺ and FAD so that the versatile and highly sensitive techniques of fluorescent spectroscopy can be used to help in understanding the structure-function relationships operating in these important molecules.

NATURAL FLUORESCENT tRNA COMPONENTS

The class of highly substituted guanosine derivatives called "Y bases"⁵⁻⁸ are the only indigenous nucleosides that have shown utility as fluorescent probes of tRNA tertiary structure. The fluorescence emission of the common nucleosides adenosine, guanosine, cytidine, and uridine are almost undetectable at neutral pH and ambient temperature. Among the modified nucleosides isolated from tRNA, the Y bases, 7-methylguanosine, 4-thiouridine, and N⁴-acetylcytidine are fluorescent.⁴ Fluorescence studies on dinucleotides and polynucleotides containing 7-methylguanosine and N⁴-acetylcytidine⁹ and on the polynucleotide composed of 4-thiouridine¹⁰ have been published, but the weak emission of these modified nucleosides has discouraged their further use as fluorescent probes of tRNA tertiary structure. By contrast, upon excitation at 315 nm, Y base shows strong fluorescence emission at 450 nm with a quantum efficiency of 7%.⁴ As a result of these fluorescence properties, Y base has been exploited in a variety of experiments designed to gain information concerning tRNA tertiary structure.^{4,11,12} These experiments will be discussed briefly for histori-

*This work was supported by Research Grant GM-05829 from the National Institutes of Health, United States Public Health Service.

cal background and to illustrate possible uses of fluorescence techniques in the investigation of tRNA tertiary structure.

Located on the 3'-end of the anticodon of the phenylalanine-specific tRNAs from several sources, the structures of the Y bases found in baker's yeast,⁵ torula yeast,⁶ and bovine liver⁷ have been reported. That the Y base resides in a hydrophobic or stacked environment within the anticodon loop is indicated by the 15-nm blue shift witnessed for the emission maximum of Y in yeast tRNA^{Phe}.¹³ From the results of fluorescence depolarization studies,¹⁴ the orientation of Y in the anticodon loop is not rigidly fixed in yeast tRNA^{Phe}. However, changes in the degree of polarization of fluorescence with increased temperature show that Y base is held within certain limits in the anticodon loop and that these limits disappear as the tRNA structure is disrupted.¹⁵ Fluorescence studies of oligonucleotides excised from yeast tRNA^{Phe} have further aided in understanding the molecular environment of the Y base in the anticodon loop.¹⁵ In investigating the mechanism of tRNA action, Y fluorescence was monitored in the presence of the complementary codon of yeast tRNA^{Phe}.¹⁷ The small blue shifts observed were used to calculate association constants for codon-anticodon binding at several temperatures.¹⁷ In addition to investigations probing the structural and dynamic properties of the anticodon region, the fluorescence of Y base has been used to monitor changes in tRNA tertiary structure. Romer *et al.*,¹⁸ combining several experimental techniques including fluorescence, have identified some five conformational transitions in the melting of yeast tRNA^{Phe}. Changes in Y fluorescence have also been found to be a sensitive means of monitoring tRNA conformational changes induced by the presence or absence of magnesium.¹⁹⁻²¹ A fourfold enhancement of fluorescence in beef liver tRNA^{Phe}¹⁹ and yeast tRNA^{Phe}²⁰ was observed over the magnesium concentration range of 0-0.4 mM. Concomitant changes in the ORD¹⁹ and CD²⁰ spectra as well as hypochromicity changes^{18,19} lend support to the proposal that this enhancement is in response to conformational changes in the tRNA. Additional studies involving the dependence of aminoacylation of yeast tRNA^{Phe} on the presence of magnesium²¹ further confirm the fact that the fluorescence enhancement represents the conversion of the tRNA^{Phe} to its biologically active form principally through the formation of a tertiary structure. That the binding of yeast Phe-tRNA synthetase to purified yeast tRNA^{Phe} in the absence of magnesium produces an enhancement of fluorescence intensity similar to that observed in the tRNA^{Phe} in the presence of magnesium also confirms the proposed conclusion.²² Similar observations involving enhancement of fluorescence in the presence of magnesium have been made by other groups.^{13,14} Still further evidence of the dependence of tRNA tertiary structure on the presence of magnesium is found in observations noted in the binding of fluorescent dyes to tRNA. Ethidium bromide was found to bind close to Y in the absence of magnesium and considerably farther away in its presence.²³ This dependence of Y fluorescence on magnesium concentration should be remembered if the fluorescence assay reported by Yoshikami *et al.*²⁴ is used. Finally, attachment of three different fluorescent dyes to the periodate-oxidized 3'-end of yeast tRNA^{Phe} has made possible the use of resonant energy transfer in determining the distance from the Y base in the anticodon loop to the -CCA 3'-terminus of yeast tRNA^{Phe}. Using Y base as the energy donor and either acriflavine, proflavinyl acetic acid hydrazide, or 9-hydrazoacridine as the energy acceptor, Beardsley and Cantor were able to determine that the fluorophores are at least 40 Å apart in solution.²⁵

FLUORESCENT ANALOGS OF NUCLEOSIDES

In the absence of naturally occurring fluorescent nucleosides, fluorescent analogs of nucleosides, because they are substrates for many nucleic acid metabolic enzymes,

Leonard & Tolman: FI

have been used as fluorescent probes containing 7-methylinosine,¹⁹ riboside,²⁷ formycin,²⁸ and 7-deaza-²⁹ the weak fluorescence emission of their usefulness as fluorescent probes for the incorporation of formycin into the 3'-terminal oligonucleotide of the anion exchange resin. Examination of the anion exchange resin at various temperatures led them to conclude that parts of the tRNA molecule.²⁶ Reaction of formycin (F) into the 3'-terminal nucleotidyl transferase.³⁰ On comparison of the tRNA^{Phe} with the tRNA^{Phe} excised from the tRNA^{Phe}, it was found that the terminal oligonucleotide of the tRNA molecule as previously proposed cleavage of the 3'-terminal ribose moiety of the incorporated formycin.³⁰ Sin deazanebularin into the 3'-terminal and Reich.³¹

CHEMICAL INTRICACIES

Chemical methods designed to label tRNA have been used to link fluorescent groups to tRNA. Attaching fluorescent markers bearing a fluorescent group to a tRNA molecule involves the formation of a covalent bond between the fluorescent group and the tRNA molecule. The choice of the fluorescent group and the periodate-oxidized 3'-terminal nucleotide of the tRNA molecule are important. As reported by this procedure, bound acriflavine, which is a fluorescent dye, was used to label the tRNA in fluorescence studies. The size and shape of the tRNA molecule, the degree of fluorescence polarization, and the induced helix-coil transition of unfractionated tRNA with mercurials,³⁵ formaldehyde,³⁶ and other reagents in experiments using Y base fluorescence were studied. Proflavinyl acetic acid hydrazide, and yeast tRNA^{Phe} using this procedure.²⁵ Methods with proflavine and ethidium bromide for labeling the anticodon loop of tRNAs, so that the interaction of tRNA with aminoacyl-tRNA synthetases could be studied by measuring fluorescence. The major drawback of the periodate-oxidized tRNA is that oxidized tRNAs cannot be used for labeling. Lynch and Schimmel^{39,40} have attached 2-naphthoxyacetic acid to the 3'-terminal nucleotide of tRNA^{Phe} and fluorescence emission of this probe for changes of the tRNA^{Phe} and was used to study the -CCA terminus and the binding of tRNA to aminoacyl-tRNA synthetases. They reported a general method for preparing fluorescently labeled tRNAs which involves modification of the 5'-phosphate of some fluorescent dyes.⁴¹ Preliminary studies of anthranilic acid and its derivatives showed that the labeled tRNA^{Phe} c

uses of fluorescence techniques in the

of phenylalanine-specific tRNAs from found in baker's yeast,⁵ torula yeast,⁶ the Y base resides in a hydrophobic or p is indicated by the 15-nm blue shift yeast tRNA^{Phe}.¹³ From the results of station of Y in the anticodon loop is changes in the degree of polarization of v that Y base is held within certain its disappear as the tRNA structure is leotides excised from yeast tRNA^{Phe} ular environment of the Y base in the ism of tRNA action, Y fluorescence entary codon of yeast tRNA^{Phe}.¹⁷ The late association constants for codon- In addition to investigations probing iticodon region, the fluorescence of Y NA tertiary structure. Romer *et al.*,¹⁸ cluding fluorescence, have identified elting of yeast tRNA^{Phe}. Changes in Y ensitive means of monitoring tRNA ice or absence of magnesium.¹⁹⁻²¹ A iver tRNA^{Phe} and yeast tRNA^{Phe} 20 on r of 0-0.4 mM. Concomitant well hypochromicity changes^{18,19} ment is in response to conformational ing the dependence of aminoacylation m²¹ further confirm the fact that the ersion of the tRNA^{Phe} to its biolog- ation of a tertiary structure. That the ified yeast tRNA^{Phe} in the absence of rescence intensity similar to that ob- gnesium also confirms the proposed enhancement of fluorescence in the her groups.^{13,14} Still further evidence on the presence of magnesium is found cent dyes to tRNA. Ethidium bromide f magnesium and considerably farther luorescence on magnesium concentra- assay reported by Yoshikami *et al.*²⁴ is rescent dyes to the periodate-oxidized he use of resonant energy transfer in the anticodon loop to the -CCA se as the energy donor and either , or 9-hydrizoacridine as the energy determine that the fluorophores are at

OF NUCLEOSIDES

escent nucleosides, fluorescent analogs many nucleic acid metabolic enzymes,

have been used as fluorescent probes of nucleic acids. Oligo- and polynucleotides containing 7-methylinosine,¹⁹ 2-aminopurine riboside,²⁶ 2,6-diaminopurine riboside,²⁷ formycin,²⁸ and 7-deazanebularin^{27,28} have been synthesized; however, the weak fluorescence emission of some analogs in polynucleotides has diminished their usefulness as fluorescent probes. Ward *et al.* have reported the enzymatic incorporation of formycin into the 3'-terminal position of rabbit liver tRNA.^{26,29} Their examination of the anion fluorescence pK and changes in fluorescence intensity with temperature led them to conclude that the terminal formycin interacts with other parts of the tRNA molecule.²⁶ Recently, Maelicke, *et al.* have reported the incorporation of formycin (F) into the 3'-terminal position of purified yeast tRNA^{Phe} using nucleotidyl transferase.³⁰ On comparison of the quenching of formycin fluorescence observed in the tRNA^{Phe} with that observed in the oligonucleotide CpApCpCpF excised from the tRNA^{Phe}, it was found that the quenching is due to stacking interactions in the terminal oligonucleotide and not to interactions with other regions of the tRNA molecule as previously proposed.³⁰ In support of this conclusion, periodate cleavage of the 3'-terminal ribose moiety of the tRNA^{Phe} restored the full fluorescence of the incorporated formycin.³⁰ Similar incorporation of the fluorescent analog 7-deazanebularin into the 3'-terminal position of tRNA has been carried out by Brdar and Reich.³¹

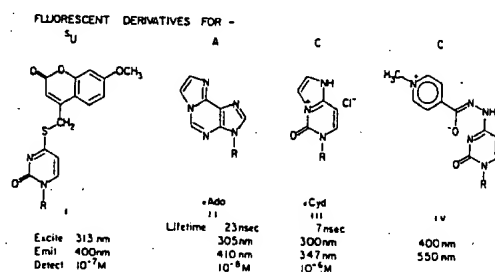
CHEMICAL INTRODUCTION OF FLUORESCENCE

Chemical methods designed to label specifically the 3'- and 5'-ends of tRNA have been used to link fluorescent groups covalently to tRNAs. A general method for attaching fluorescent markers bearing primary amino groups to the 3'-end of the tRNA molecule involves the formation of a Schiff base between the fluorescent dye of choice and the periodate-oxidized 3'-terminal ribose unit.³²⁻³⁷ Churchich, who first reported this procedure, bound acriflavine to the 3'-end of crude yeast tRNA and used the labeled tRNA in fluorescence polarization studies to obtain data concerning the size and shape of the tRNA molecule in solution.³² Using a similar procedure to label crude *E. coli* tRNA with acriflavine,³³ Millar and coworkers monitored the degree of fluorescence polarization of the labeled tRNA to follow the thermally-induced helix-coil transition of unfractionated tRNA before³⁴ and after treatment with mercurials,³⁵ formaldehyde,³⁶ and acrylonitrile.³⁷ In previously described experiments using Y base fluorescence in resonant energy transfer studies, acriflavine, proflavinyl acetic acid hydrazide, and 9-hydrizoacridine were bound to the 3'-end of yeast tRNA^{Phe} using this procedure.²⁵ Pachmann, *et al.* have also applied fluorescence methods with proflavine and ethidium bromide placed in the D loop or the anticodon loop of tRNAs, so that the interaction of these purified tRNAs with their cognate synthetases could be studied by means of fluorescence polarization techniques.³⁸ The major drawback of the periodate method in introducing fluorescent probes into tRNA is that oxidized tRNAs cannot be aminoacylated. To avoid this drawback, Lynch and Schimmel^{39,40} have prepared fluorescently labeled tRNA by attaching 2-naphthoxyacetic acid to the amino group of yeast isoleucyl-tRNA^{Ile}. The fluorescence emission of this probe proved to be very sensitive to the structural changes of the tRNA^{Ile} and was used to study the kinetics of base ionization in the -CCA terminus and the binding of magnesium to tRNA^{Ile}. Yang and Söll have reported a general method for preparing tRNAs specifically labeled at the 5'-end that involves modification of the 5'-phosphate with the phosphomorpholidate derivatives of some fluorescent dyes.⁴¹ Preliminary studies of *E. coli* tRNA^{Met} labeled with derivatives of anthranilic acid and of dimethylaminonaphthalenesulfonic acid showed that the labeled tRNA^{Met} could be aminoacylated and that there is

significant interaction between the fluorescent probes and the tRNA macromolecule.⁴¹

Another general chemical method for introducing fluorescent probes into tRNA involves the insertion of fluorescent dyes bearing primary amino groups into the positions of selectively removed modified bases.^{42,43} Dihydrouracil, 7-methylguanine, and Y base can be specifically excised from tRNA, leaving the aldehyde at the C₁ carbon of the ribose available for Schiff base formation with the fluorescent dye.⁴³ Wintermeyer and Zachau have reported the insertion of proflavine and ethidium bromide into various positions of tRNA^{Phe} and tRNA^{Ser} from yeast using this procedure.⁴³ The main drawback of this method is that insertion of dye into every available tRNA molecule is difficult to achieve. Friest *et al.* have utilized this method to insert 3-methyl-2-benzothiazolone hydrazone into Y-excised yeast tRNA^{Phe}; however, the product was only weakly fluorescent.⁴⁴ The labeled tRNAs can be aminoacylated so that they can be used in studying tRNA-synthetase interactions.⁴³

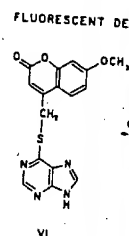
Fluorescent groups have also been introduced into tRNAs by reaction with the purine and pyrimidine bases. Chemical modification of tRNA with N-acetoxy-2-acetylaminofluorene, which reacts with guanosine at position 8, has been carried out,⁴⁴⁻⁴⁷ but the fluorescence emission of the fluorophore in the modified tRNA is too weak to be useful as such. The reaction of 4-bromomethyl-7-methoxy-2-oxo-2H-benzopyran (BMB)[†] with 4-thiouridine produces a fluorescent derivative (I,



R = ribosyl) with the properties shown.⁴⁸ The fluorescence is caused by emission from the excited coumarin ring, and the pyrimidine ring has little, if any, effect on the fluorescence. This example from our laboratory of a fluorescent reagent giving a product having the same unit responsible for the fluorescence typifies the case in which it is obviously necessary to remove unreacted reagent completely in order to be assured that the fluorescence properties recorded are alone due to the product and its environment. A second and possibly "cleaner" approach to the introduction of fluorescence has been used in our laboratory that involves the use of a nonfluorescent reagent, usually a small molecule. This approach is exemplified by the reaction of chloroacetaldehyde specifically and quantitatively with adenosine and cytidine to form fluorescent derivatives (II, III, R = ribosyl) having different emission characteristics, as shown, making them easily distinguishable. In these cases, the chloroacetal-

[†] Abbreviations following the IUPAC-IUB Commission on Biochemical Nomenclature 1971 recommendations (J. Mol. Biol. 55: 299) are used throughout. BMB reagent is 4-bromomethyl-7-methoxy-2-oxo-2H-benzopyran. The abbreviation "ε" stands for etheno, so that ε-adenosine (εAdo) is 3-β-D-ribofuranosylimidazo[2,1-i]purine (1,N⁶-etheno-adenosine), and ε-cytidine (εCyd) is 5,6-dihydro-5-oxo-6-β-D-ribofuranosylimidazo[2,1-c]pyrimidine (3,N⁴-ethenocytidine).⁶³ The corresponding nucleotide derivatives are abbreviated by adding "ε" before the approved abbreviations, i.e., εATP, εCTP, εADP, εAMP, εcAMP, etc.

dehyde initial reagent does no scavenging. Products related nonfluorescent, isonicotinic specifically to produce a zwitter emission maxima shown.⁴⁹ The emission maximum which may energy transfer from I to IV, if suitable distance in a tRNA, is 400 nm, and excitation of IV by reaction of BMB with *E. coli* tRNA modification does not affect tRNA synthetase.⁵⁰ Another 2-thio-5-(N-methylaminomethyl) tRNA^{Glu}, is reported to react al dine reacts more slowly, at modification reaction with che specifically labeling the 3'-end a cent probes have been prepared transfer determinations of intrat for the apparent distances betw (38 Å), pseudouridine to 3' end in *E. coli* tRNA^{Met} and between 3' end (> 65 Å) of *E. coli* tRNA has been its use in the conve thioguanine) to the correspondi



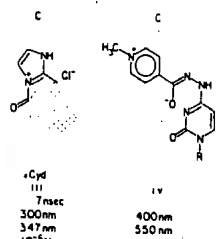
fluorescent tagging of the parer following their cellular fate. In ac to tRNA tertiary structure, fluo studying the intramolecular inte nicotinamide-adenine dinucleotid. Exploiting the fluorescence of the flavin coenzyme (FAD) and of the the nicotinamide coenzyme (NAD been demonstrated in these two c fluorescence lifetimes, quantum ef

FLUORESC

In incorporating fluorescence coenzymes NAD⁺ and FAD, the r with adenosine and cytidine to g dimension for studies of these l

roducing fluorescent probes into tRNA bearing primary amino groups into the modified bases.^{42,43} Dihydrouracil, 7-ically excised from tRNA, leaving the available for Schiff base formation with Zachau have reported the insertion of ious positions of tRNA^{Phe} and tRNA^{Ser} n drawback of this method is that inser-ecule is difficult to achieve. Friest *et al.* yl-2-benzothiazolone hydrazine into Y-uct was only weakly fluorescent.⁴⁴ The at they can be used in studying tRNA-

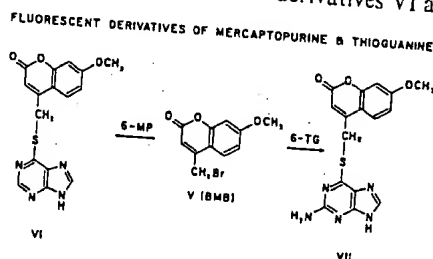
duced into tRNAs by reaction with the modification of tRNA with N-acetoxy-2-anosine at position 8, has been carried he fluorophore in the modified tRNA is of 4-bromomethyl-7-methoxy-2-oxo-2H- produces a fluorescent derivative (I,



The fluorescence is caused by emission from the imidinium ring. The imidinium ring has little, if any, effect on the reactivity of a fluorescent reagent giving a false positive. The case for the fluorescence typifies the case in which the fluorescent reagent completely in order to be recorded are alone due to the product and the "leaner" approach to the introduction of fluorescence that involves the use of a nonfluorescent reagent. This approach is exemplified by the reaction of the reagent with adenosine and cytidine (to give the fluorescent product) having different emission characteristics. In these cases, the chloroacetal-

Commission on Biochemical Nomenclature 1971) throughout. BMB reagent is 4-bromomethyl- ϵ -L-thio stands for etheno, so that ϵ -adenosine (1, N^6 -ethenoadenosine), and ϵ -cytidine (2,5-imidazo[2,1-*c*]pyrimidine (3, N^4 -ethenocytosine) are abbreviated by adding " ϵ " before the ADP, ϵ AMP, ϵ cAMP, etc.

dehydrate initial reagent does not pose the problem that a fluorescent reagent does for scavenging. Products related to II and III are considered in detail below. Also nonfluorescent, isonicotinic acid hydrazide methiodide reacts with cytidine specifically to produce a zwitterionic fluorescent product IV with the absorption and emission maxima shown.⁴⁹ The environment has an effect on the wavelength of the emission maximum which may be of advantage. Also a possibility are applications of energy transfer from I to IV, if such derivatives of ³H and C would be located within suitable distance in a tRNA, in keeping with excitation of I at 313 nm, emission at 400 nm, and excitation of IV by this emission, with ultimate emission at 550 nm. The reaction of BMB with *E. coli* tRNA^{Met} specifically modifies the 4-thiouridine, and the modification does not affect the aminoacylation by the homologous aminoacyl-tRNA synthetase.⁵⁰ Another thionucleoside, but a 2-thiouridine type, namely 2-thio-5-(N-methylaminomethyl)uridine, which occurs in the anticodon of *E. coli* tRNA^{Glu}, is reported to react almost quantitatively with BMB,⁵⁰⁻⁵² while pseudouridine reacts more slowly, at N-1, with the reagent.^{51,52} Combining the BMB modification reaction with chemical methods for replacing dihydrouracil and for specifically labeling the 3'-end and 5'-end, five tRNAs bearing two different fluorescent probes have been prepared by Yang and Söll⁵² for use in singlet-singlet energy transfer determinations of intramolecular distances. Values could then be calculated for the apparent distances between the 3' and 5' end (24 Å), 4-thiouridine to 3' end (38 Å), pseudouridine to 3' end (55 Å), and pseudouridine to dihydrouridine (36 Å) in *E. coli* tRNA^{Met} and between the 2-thiouridine derivative to dihydrouridine and the 3' end (> 65 Å) of *E. coli* tRNA^{Glu}.⁵² Another application for the BMB reagent (V) has been its use in the conversion of 6-MP (6-mercaptopurine) and 6-TG (6-thioguanine) to the corresponding fluorescent derivatives VI and VII. We hope this



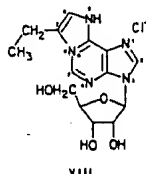
fluorescent tagging of the parent anticancer compounds may be of some use in following their cellular fate. In addition to their usefulness in investigations relating to tRNA tertiary structure, fluorescence techniques have been of great value in studying the intramolecular interactions present in the dinucleotide coenzymes nicotinamide-adenine dinucleotide (NAD⁺) and flavin-adenine dinucleotide (FAD). Exploiting the fluorescence of the isoalloxazine moiety in the oxidized form of the flavin coenzyme (FAD) and of the dihydropyridinium moiety in the reduced form of the nicotinamide coenzyme (NADH), the existence of intramolecular complexes has been demonstrated in these two oxidation-reduction dinucleotide coenzymes using fluorescence lifetimes, quantum efficiencies, and polarization studies.⁵³⁻⁶⁰

FLUORESCENT A AND C DERIVATIVES

In incorporating fluorescence into specific nucleic acid bases in tRNA and the coenzymes NAD⁺ and FAD, the reaction of chloroacetaldehyde in aqueous solution with adenosine and cytidine to give fluorescent products (II, III) affords another dimension for studies of these biological systems. Kochetkov *et al.*⁶¹ initially

reported the reaction of chloroacetaldehyde with 9-methyladenine and 1-methylcytosine, and this led us to examine further the reaction of chloroacetaldehyde with the nucleosides adenosine and cytidine with the hope and subsequent realization that the products would be fluorescent.⁶² While 3,*N*⁴-ethenocytidine (III, R = ribosyl)⁶² has had limited use as a fluorescent probe because it is fluorescent only in the protonated form, the fluorescence properties of 1,*N*⁶-ethenoadenosine⁶² and its nucleotide derivatives^{62,63} have been exploited in numerous investigations of biological systems.

The chloroacetaldehyde modification reaction offers great advantages in rendering nucleic acid bases in biological systems fluorescent, since the reaction meets the major goal of being capable of being carried out in aqueous media under mild conditions of pH and temperature.^{62,63} The reaction at 37° proceeded rapidly at the optimum pH of 4.5 for adenosine and 3.5 for cytidine.^{62,64} The formation of the etheno bridge, since it is symmetrical, does not establish the direction of its incorporation, although the formal mechanism involving reaction of the α -carbon with N-1 and the aldehyde carbon with N⁶ envisaged in the case of adenosine was corroborated by deuterium-labeling studies and nmr analysis.⁶³ X-ray analysis of the product of the reaction of α -chlorobutyraldehyde established its structure as 7-ethyl-3- β -D-ribofuranosylimidazo[2,1-*i*]purine hydrochloride (VIII), which did establish

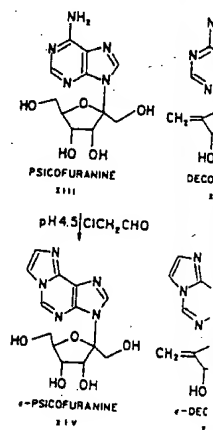


that the N-1 of adenosine had displaced the chlorine from the α -carbon of the aldehyde.⁶⁵ The crystal structure also showed the excellent "stacking" properties of the ϵ -adenine rings, for in the nonpolar region of the monoclinic monohydrate there are infinite stacks of overlapping ϵ -adenine rings with alternate ring separations of 3.344 and 3.324 Å. The bare ring system, imidazo[2,1-*i*]purine, was first formed by acid treatment of 6-formylmethyladenine, and its pyrimidine ring opening and reclosure have been effected by Shaw and Smallwood.⁶⁶ Yip and Tsou⁶⁷ modified the 1,*N*⁶-ethenoadenosine structure (II, R = ribosyl) by basic hydrolysis to remove the original C-2 of the adenine moiety followed by treatment with nitrous acid to give 2-aza-1,*N*⁶-ethenoadenosine (IX, R' = H). The sequential treatments with base and nitrous acid are too harsh to make this further modification of ϵ -adenosine applicable to intact RNAs, but the structures realized at the nucleoside and nucleotide level are of interest because of the long wavelength of fluorescence emission (494 nm) of compounds of type IX.

Other α -haloaldehydes in general are less water-soluble and react more slowly than chloroacetaldehyde with adenosine and its derivatives. These include α -chloropropionaldehyde,⁶⁴ α -chlorobutyraldehyde,^{64,65} α -bromovaleraldehyde,⁶⁸ and 2-bromo-2-phenylacetaldehyde,⁶⁸ leading to 7-methyl, 7-ethyl-, 7-propyl-, and 7-phenyl-1,*N*⁶-ethenoadenosine derivatives, respectively. The chloroacetaldehyde reaction has also been applied to analogs of cAMP having CH₂ in place of the 5'-O (X). Another source of 7-alkyl-1,*N*⁶-ethenoadenosine derivatives is in the mild oxidative cyclization of N⁶-(Δ^2 -isopentenyl)adenosine.⁶⁹ 8-Phenyl-1,*N*⁶-ethenoadenosine derivatives have been synthesized using α -bromoacetophenone.^{64,68,70} 7,8-Dimethylimidazo[2,1-*i*]purine, with double substitution on the new ring, was prepared from β -acetylvinyltriphenylphosphonium bromide and adenine.⁷¹

Leonard & Tolman: F

The chloroacetaldehyde reactant, the deoxyribosyl derivatives arabinosyl- ϵ -cytosine (XII), in cc the National Cancer Institute, with resistant to deaminases. Indeed, deaminase and XII to cytidine d protection did not lead to the de tumor system.⁷⁴ Among the many the chloroacetaldehyde reaction with cofuranine (XIII \rightarrow XIV), decoyir



one of the products of formycin reported the formation of ϵ -tuberc side antibiotics toyocamycin and s: 1,*N*⁶-etheno derivatives.⁷⁵ The flu these " ϵ " derivatives were found to in neutral aqueous solution.^{62,63}

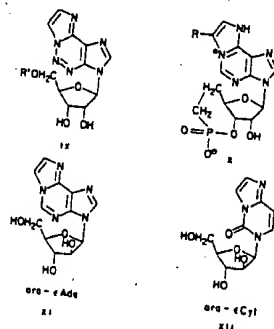
SPECIES RESPONSIBLE FOR THE

In an examination of the sp 1,*N*⁶-ethenoadenine moiety in all o obtained the fluorescence lifetimes

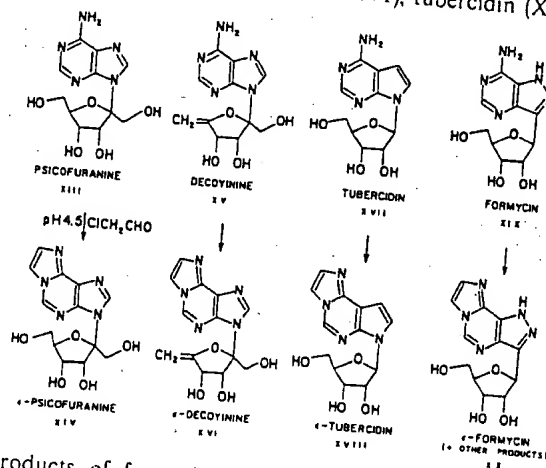
ion offers great advantages in rendering fluorescent, since the reaction meets the following criteria: (1) it takes place in aqueous media under mild conditions; (2) the reaction at 37° proceeded rapidly at the concentration of 10⁻³ M for cytidine.^{62,64} The formation of the adduct does not establish the direction of its reaction, since the reaction involving reaction of the α -carbon of the nucleoside was envisaged in the case of adenosine was confirmed by nmr analysis.⁶³ X-ray analysis of the adducts of cytosine established its structure as 7-ethyl-2-chloride (VIII), which did establish

the excellent "stacking" properties of the monoclinic monohydrate there are large interlayer separations of 3.4 Å. The 2,6-diamino-4-azopyrimidin-5(1H)-one, which is a purine, was first formed by the condensation of 2,6-diaminopyrimidine with formaldehyde and formaldehyde. Yip and Tsou⁶⁷ modified the procedure by basic hydrolysis to remove the formaldehyde and then treatment with nitrous acid to give 2,6-diamino-4-azopyrimidin-5(1H)-one. Sequential treatments with base and formaldehyde followed by modification of ϵ -adenosine applied to the nucleoside and nucleotide level of fluorescence emission (494 nm) of

water-soluble and react more slowly than its derivatives. These include glyceraldehyde,^{64,65} α-bromovaleraldehyde,⁶⁸ and 7-methyl-, 7-ethyl-, 7-propyl-, and 7-octyl-, respectively. The chloroacetaldehyde AMP having CH₂ in place of the 5'-O ribose derivatives is in the mild oxidation state.⁶⁹ 8-Phenyl-1,N⁶-ethenoadenosine, 2,6-dichloro-2,6-dimethyl-4-nitrophenol, 2,6-dibromoacetophenone,^{64,68,70} 7,8-dibromoacetophenone, and adenine,⁷¹ were substituted on the new ring, was also used.



The chloroacetaldehyde reaction has been used with other nucleosides, for example, the deoxyribose derivatives.⁷² We have made arabinosyl- ϵ -adenine (XI) and arabinosyl- ϵ -cytosine (XII), in cooperation with the Drug Development Branch of the National Cancer Institute, with the expectation that these compounds would be resistant to deaminases. Indeed, they were resistant, XI to calf duodenal adenosine deaminase and XII to cytidine deaminase of *E. coli*;⁷³ however, this etheno bridge protection did not lead to the development of any appreciable activity in the L1210 tumor system.⁷⁴ Among the many adenine-related antibiotics, we have obtained by the chloroacetaldehyde reaction well-characterized etheno-bridged derivatives of psicofuranine (XIII \rightarrow XIV), decoyinine (XV \rightarrow XVI), tubercidin (XVII \rightarrow XVIII), and



one of the products of formycin (XIX \rightarrow XX). Schramm and Townsend have reported the formation of *ε*-tubercidin (XVIII) and, from the closely related nucleoside antibiotics toycamycin and sangivamycin, the formation of the corresponding 1,*N*⁶-etheno derivatives.⁷⁵ The fluorescence emission characteristics of all three of these "*ε*" derivatives were found to be similar to those of 1,*N*⁶-ethenoadenosine (II) in neutral aqueous solution.^{62,63}

SPECIES RESPONSIBLE FOR THE FLUORESCENCE OF 1,N⁶-ETHENOADENOSINE

In an examination of the species responsible for the fluorescence of the 1,N⁶-ethenoadenine moiety in all of its chloroacetaldehyde-modified derivatives, we obtained the fluorescence lifetimes, quantum efficiencies, and emission spectra of

ϵ AMP in aqueous solution over the pH range 1.5–12.0, which were indicative that only one fluorescent emitting species exists, namely, the unprotonated form.⁷⁶ The loss at low pH of fluorescence emission at 415 nm from the neutral 1,*N*⁶-ethenoadenosine fluorophore is due to the conversion of the fluorescent unprotonated form to the nonfluorescent protonated form by protonation at N-9 (see VIII for numbering). The observation that the fluorescence quantum efficiency for ϵ -9-propyladenine (II, R = propyl) in anhydrous dioxane, where it cannot acquire a proton in the excited state, is 86% that of ϵ AMP at pH 6.8 in aqueous solution provided direct evidence that the unprotonated form of the ϵ -adenine fluorophore is responsible for the fluorescence emission.⁷⁶ The useful fluorescence properties of 1,*N*⁶-ethenoadenosine have been summarized as: 1) long wavelength uv absorption which allows excitation outside the range of absorption of proteins and most nucleic acids; 2) intense fluorescence at 415 nm which allows its detection in the presence of protein; 3) a quantum yield of about 0.6 which allows ready detection at concentrations below 10^{-8} M; 4) long fluorescence lifetime (23 nsec for ϵ AMP) which allows depolarization studies of 1,*N*⁶-ethenoadenosine fluorescence from nucleotide derivatives bound to molecules as large as 250,000 Daltons, and 5) small structural change to adenosine which allows the biological activity of modified coenzymes to be preserved to a considerable extent with some enzymes.⁶³

ACTIVITY OF 1,*N*⁶-ETHENOADENOSINE NUCLEOTIDES IN BIOLOGICAL SYSTEMS

In ascertaining the activity of 1,*N*⁶-ethenoadenosine nucleotides in biological systems, ϵ AMP, ϵ ADP, ϵ ATP, and ϵ cAMP (II, R variant) have been substituted for the corresponding adenosine nucleotides in various enzyme systems.^{63,68,70,77–87} The ability of the 1,*N*⁶-ethenoadenosine nucleotides to act as substrates in these systems was found to depend on the specificity of the enzyme-binding site and varied from no activity in some cases (ϵ ATP with firefly luciferase⁷⁸) to full activity in others (ϵ ATP with myosin ATPase⁷⁷). Binding studies of ϵ ADP to pyruvate kinase,⁸⁸ to myosin, H-meromyosin, and subfragment one,⁸⁹ and to a mitochondrial ATPase,⁹⁰ and of ϵ ATP to pyruvate kinase,⁸⁸ H-meromyosin,⁹¹ and aspartate transcarbamylase⁹² have exploited the useful fluorescent properties of 1,*N*⁶-ethenoadenosine nucleotide derivatives in gaining more detailed information concerning these enzyme systems. It should be recognized that in cases where the ϵ -nucleotide shows appreciable activity with a particular enzyme system, it can be concluded that the 1,*N*⁶ region is not required for binding. Conversely, when activity is absent, the 1,*N*⁶ region may be required for binding or at least there must be no molecular protrusion in this part of the molecule. At all times, great care must be taken to obtain pure ϵ -nucleotide, so that the activity of any trace of the normal cofactor still present will not be mistaken for possible activity of the chloroacetaldehyde product. In further utilization of the intense fluorescence of 1,*N*⁶-ethenoadenosine, fluorometric assays^{93,94} and a spray reagent⁹⁵ have been developed for the detection of adenosine derivatives.

ETHENO DERIVATIVES AT THE OLIGO-, POLY- AND DINUCLEOTIDE LEVELS

The properties of 1,*N*⁶-ethenoadenosine and 3,*N*⁴-ethenocytidine have also been investigated at the oligo- and polynucleotide levels. Cytidylyl(3' \rightarrow 5')uridine (CpU) has been modified with chloroacetaldehyde by Kochetkov and coworkers,⁶⁴ who found that no hydrolysis of the phosphodiester linkage occurred during the course of

Leonard & Tolman: Fl

the reaction. The modified dinucleoside the action of the endonuclease poly(1,*N*⁶-ethenoadenylic acid) (poly(ϵ C)) have been synthesized by cleotide phosphorylase,^{96,97} and to nuclease action than their unmodified nor 3,*N*⁴-ethenocytidine can form hydrogen bonding sites are masked complex with poly(U), and poly(ϵ C) substitution of 20% or less of the chloroacetaldehyde-treated poly(A) and trihelical species characteristic formation of poly(A) with poly(U). than one-seventh that of ϵ AMP, with Concerning the quenching of the 1 tions between bases in poly(ϵ A), S ching in poly(A) containing 80% 1 degrees of substitution and having 1,*N*⁶-ethenoadenosine are more adenosine-1,*N*⁶-ethenoadenosine in chloroacetaldehyde, and the project investigated.⁹⁹ To introduce 1, Zachau^{100,101} utilized tRNA nucleic ϵ AMP moiety from ϵ ATP into the tRNA^{ser} from baker's yeast, but later unfortunately was not actually incorporated. Nicotinamide 1,*N*⁶-ethenoadenosine 1,*N*⁶-ethenoadenosine dinucleotide (el tion of chloroacetaldehyde with NA reasonable range of activity as substrate enzymes. ϵ FAD shows extremely efficient to the isalloxazine moiety. Determin provided data that can be used to closed (intramolecularly complexed) neutral aqueous solution, it was four stacked form.

CHLOROACETALDEHYDE-M

The twelve possible dinucleosides with adenosine, cytidine, guanosine, 1,*N*⁶-ethenoadenosine and 3,*N*⁴-ethenocytidine, and chloroacetaldehyde.¹⁰⁷ Those dinucleosides are fluorescent in neutral solution, with not, since 3,*N*⁴-ethenocytidine is fluorescent in chloroacetaldehyde modification in general resistant to nucleolytic cleavage.^{64,96} were completely resistant to the action of ϵ ApN and ϵ CpN were highly resistant quenching parameters were determined lifetimes and quantum efficiencies

.5-12.0, which were indicative that rely, the unprotonated form.⁷⁶ The at 415 nm from the neutral onversion of the fluorescent unpro-orm by protonation at N-9 (see VIII scence quantum efficiency for ϵ -9-ioxane, where it cannot acquire a MP at pH 6.8 in aqueous solution orm of the ϵ -adenine fluorophore is e useful fluorescence properties of : 1) long wavelength uv absorption orption of proteins and most nucleic llows its detection in the presence of llows ready detection at concentra- re (23 nsec for ϵ AMP) which allows fluorescence from nucleotide deriva- tons, and 5) small structural change vity of modified coenzymes to be izymes.⁶³

EOTIDES IN BIOLOGICAL SYSTEMS

adenosine nucleotides in biological R variant) have been substituted for rious enzyme systems.^{63,68,70,77-87} otide act as substrates in these if the yme-binding site and varied luciferase⁷⁸) to full activity in others s of ϵ ADP to pyruvate kinase,⁸⁸ to ⁹ and to a mitochondrial ATPase,⁹⁰ H-meromyosin,⁹¹ and aspartate useful fluorescent properties of gaining more detailed information recognized that in cases where the particular enzyme system, it can be or binding. Conversely, when activity binding or at least there must be no ible. At all times, great care must be activity of any trace of the normal possible activity of the chloroacet- of the intense fluorescence of ⁴ and a spray reagent⁹⁵ have been itives.

Y- AND DINUCLEOTIDE LEVELS

1 3,N⁴-ethenocytidine have also been vels. Cytidylyl(3' \rightarrow 5')uridine (CpU) y Kochetkov and coworkers,⁶⁴ who linkage occurred during the course of

the reaction. The modified dinucleoside phosphate ϵ CpU proved to be resistant to the action of the endonuclease pancreatic RNase A. The polynucleotides poly(1,N⁶-ethenoadenylic acid) (poly(ϵ A)) and poly(3,N⁴-ethenocytidylic acid) (poly(ϵ C)) have been synthesized from ϵ ADP and ϵ CDP, respectively, using polynucleotide phosphorylase,^{96,97} and both polymers were found to be more resistant to nuclease action than their unmodified counterparts. Neither 1,N⁶-Ethenoadenosine nor 3,N⁴-ethenocytidine can form Watson-Crick base pairs, since the necessary hydrogen bonding sites are masked by the etheno bridge. Thus, poly(ϵ A) does not complex with poly(U), and poly(ϵ C) does not complex with poly(I).⁹⁶ However, substitution of 20% or less of the adenosine residues with 1,N⁶-ethenoadenosine in chloroacetaldehyde-treated poly(A) was found not to block the formation of the bi- and trihelical species characteristic of the acid form of poly(A) or the complex formation of poly(A) with poly(U).⁹⁸ The fluorescence intensity of poly(ϵ A) was less than one-seventh that of ϵ AMP, while poly(ϵ C) showed no significant fluorescence.⁹⁶ Concerning the quenching of the 1,N⁶-ethenoadenosine fluorescence by the interactions between bases in poly(ϵ A), Steiner and coworkers⁹⁸ have noted more quenching in poly(A) containing 80% 1,N⁶-ethenoadenosine than in poly(A) with lower degrees of substitution and have suggested that homologous interactions of 1,N⁶-ethenoadenosine are more efficient in quenching fluorescence than adenosine-1,N⁶-ethenoadenosine interactions.⁹⁸ DNA has also been treated with chloroacetaldehyde, and the properties of the fluorescent product have been investigated.⁹⁹ To introduce 1,N⁶-ethenoadenosine into tRNA, Hertz and Zachau^{100,101} utilized tRNA nucleotidyltransferase in an attempt to incorporate the ϵ AMP moiety from ϵ ATP into the 3'-terminal position of purified tRNA^{Phe} and tRNA^{Ser} from baker's yeast, but later concluded that the ϵ AMP moiety of ϵ ATP unfortunately was not actually incorporated into these tRNAs.¹⁰² Nicotinamide 1,N⁶-ethenoadenine dinucleotide (ϵ NAD⁺)^{103,104} and flavin 1,N⁶-ethenoadenine dinucleotide (ϵ FAD)^{105,106} have been synthesized by the reaction of chloroacetaldehyde with NAD⁺ and FAD, respectively, and both showed a reasonable range of activity as substitutes for the normal coenzymes with a variety of enzymes. ϵ FAD shows extremely efficient energy transfer from the ϵ -adenine moiety to the isoalloxazine moiety. Determination of the fluorescence yields and lifetimes provided data that can be used to generate the relative proportions of open and closed (intramolecularly complexed) conformations,⁵⁵ and, in the case of ϵ FAD in neutral aqueous solution, it was found to exist ca. 90% as an internally complexed or stacked form.

CHLOROACETALDEHYDE-MODIFIED DINUCLEOSIDE PHOSPHATES

The twelve possible dinucleoside phosphates combining adenosine and cytidine with adenosine, cytidine, guanosine, and uridine have been converted to the 1,N⁶-ethenoadenosine and 3,N⁴-ethenocytidine analogs by reaction with chloroacetaldehyde.¹⁰⁷ Those dinucleoside phosphates containing 1,N⁶-ethenoadenosine are fluorescent in neutral solution, while those containing 3,N⁴-ethenocytidine are not, since 3,N⁴-ethenocytidine is fluorescent only in the protonated form. Chloroacetaldehyde modification in general renders the dinucleoside phosphates more resistant to nucleolytic cleavage.^{64,96} Dinucleoside phosphates of the form ϵ CpN ϵ ApN and ϵ CpN were highly resistant to the action of RNase T₂. Static and dynamic quenching parameters were determined from the values measured for the fluorescence lifetimes and quantum efficiencies of the seven 1,N⁶-ethenoadenosine dinu-

cleoside phosphates. From the fluorescence quenching parameters, it was possible to determine the proportion of internally complexed or folded conformations versus open or unfolded conformations at 25°, as had been done in the case of FAD.¹⁰⁶ The figures obtained for the degree of internal association, in 5×10^{-5} M aqueous solution, were as follows: ϵ ApeA, 68%; ϵ ApG, 62%; GpeA, 72%; ϵ ApeC, 58%; ϵ CpeA, 15%; ϵ ApU, 44%; UpeA, 28% (all $\pm 5\%$). Guanosine and 1,N⁶-ethenoadenosine participate equally well in stacking interactions in the dinucleoside phosphates, and in general greater intramolecular association was observed in the dinucleoside phosphates containing purines than those containing pyrimidines. The sequence effects on intramolecular association observed in the 1,N⁶-ethenoadenosine dinucleoside phosphates are identical with those observed for their unmodified counterparts.¹⁰⁸ Thus, the fluorescence-quenching parameters of UpeA and ϵ CpeA indicate a lower degree of base-base interaction than in their ϵ ApU and ϵ ApeC partners. In order to investigate further the intramolecular interactions of ϵ CpeA in comparison with those in ϵ ApeC, we determined the circular dichroic spectra in neutral solution of ϵ ApeC, ϵ CpeA, and a mixture of the component nucleosides. The CD spectrum of ϵ ApeC indicates a large change from the summation of the curves for the individual components and thus indicative of stacking.¹⁰⁸ By contrast, the CD spectrum

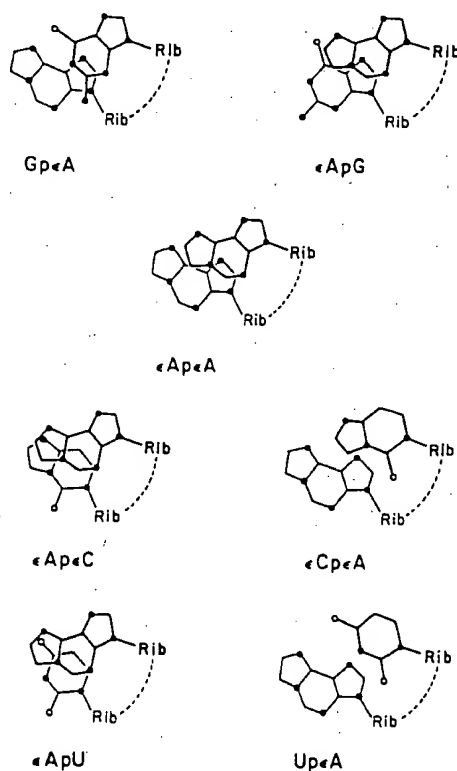


FIGURE 1. Conformations of the seven 1,N⁶-ethenoadenosine dinucleoside phosphates as viewed normal to the planes of the bases and drawn as though they were part of an RNA-11 helix. (By permission of the publishers of Biochemistry.¹⁰⁷)

Leonard & Tolman:

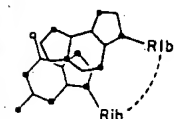
of ϵ CpeA is closer to that of the effect and indicating that the conformation of ϵ CpeA in buffered solution is closer to that of ϵ ApeC. The patterns of base-base interactions of the dinucleoside phosphates, as pictured in Figure 1, are shown for a section of the RNA-11 helix.¹¹⁰ The conformations shown for these compounds in solution, as pictured in Figure 1, are shown for a section of the RNA-11 helix. The conformations shown for these compounds in solution, as pictured in Figure 1, are shown for a section of the RNA-11 helix. The conformations shown for these compounds in solution, as pictured in Figure 1, are shown for a section of the RNA-11 helix.

Although extension of the results to predict the conformation of unmodified counterparts is not possible, the extent of complex formation is not a limit of the extent of complex formation. Moreover, the fluorescence quenching of the 1,N⁶-ethenoadenosine dinucleoside phosphates is not a direct measure of the extent of complex formation. The quenching of the 1,N⁶-ethenoadenosine dinucleoside phosphates is not a direct measure of the extent of complex formation.

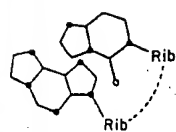
FLUOR

We mentioned earlier the fluorescence quenching of the 1,N⁶-ethenoadenosine dinucleoside phosphates. We have sought to provide synthetic controls for the fluorescence quenching of the 1,N⁶-ethenoadenosine dinucleoside phosphates. We have sought to provide synthetic controls for the fluorescence quenching of the 1,N⁶-ethenoadenosine dinucleoside phosphates. We have sought to provide synthetic controls for the fluorescence quenching of the 1,N⁶-ethenoadenosine dinucleoside phosphates.

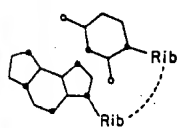
anching parameters, it was possible to fixed or folded conformations versus been done in the case of FAD.¹⁰⁶ The ciation, in 5×10^{-5} M aqueous solu- %; GpA, 72%; ϵ ApC, 58%; ϵ CpA, uanosine and 1,N⁶-ethenoadenosine s in the dinucleoside phosphates, and was observed in the dinucleoside pho- ing pyrimidines. The sequence effects a 1,N⁶-ethenoadenosine dinucleoside for their unmodified counterparts.¹⁰⁸ of UpA and ϵ CpA indicate a lower ApU and ϵ ApC partners. In order to tions of ϵ CpA in comparison with ichroic spectra in neutral solution of ent nucleosides. The CD spectrum of nation of the curves for the individual ¹⁰⁸ By contrast, the CD spectrum



Rib



ϵ CpA



UpA

ethenoadenosine dinucleoside phosphates as n as though they were part of an RNA-11 istry.¹⁰⁷

of ϵ CpA is closer to that of the summed component parts, showing no induced CD effect and indicating that the contribution of dissymmetrical stacked conformations to ϵ CpA in buffered solution is very small. For greater insight concerning the patterns of base-base interactions allowed by the ribose-phosphate-ribose backbone of the dinucleoside phosphate, each of the seven 1,N⁶-ethenoadenosine dinucleoside phosphates is pictured in FIGURE 1 in the proposed conformation of the dinucleoside phosphate as if it were a section of the 11-fold RNA helix described by Arnott.¹⁰⁹ The conformations shown for the 1,N⁶-ethenoadenosine dinucleoside phosphates based on the RNA-11 helix¹¹⁰ are taken as reasonable working approximations for these compounds in solution, at the same time bearing in mind that the dinucleoside phosphates have greater freedom of orientation either toward more extensive overlap or out of the helical conformations pictured in FIGURE 1. As is suggested in FIGURE 1, the ribose-phosphate-ribose backbone allows greater interaction between the 1,N⁶-ethenoadenosine and its neighboring nucleoside in ϵ ApC and ϵ ApU than in ϵ CpA and UpA, respectively, and this greater allowed interaction would account for the internal association observed. Possibly the conformation of ϵ CpA in aqueous solution is not helical but instead is similar to those observed in crystalline UpA in which the two rings do not overlap.^{111,112} The adoption of this type of conformation in ϵ CpA, which is only about 0.5 kcal/mole higher in energy than the lowest-energy helical conformation according to potential energy calculations (for UpA¹¹³), may be invoked to account for the minimal interaction between the bases in ϵ CpA.

Although extension of the results obtained for the modified fluorescent dinucleoside phosphates to predict the degree of internal association present in their unmodified counterparts is not strictly valid, the degrees of association found for the seven 1,N⁶-ethenoadenosine dinucleoside phosphates probably represent an upper limit of the extent of complex formation in the corresponding unmodified compounds. Moreover, the fluorescence technique has permitted these results to be obtained for extremely dilute solutions (5×10^{-5} M). The results can be used directly in studies involving chloroacetaldehyde-treated oligonucleotides and tRNA. The quenching of the 1,N⁶-ethenoadenosine fluorescence due to stacking interactions in the chloroacetaldehyde-treated tRNA promises to be useful in monitoring the disruption of tertiary structure. These results are valuable in our further application of the chloroacetaldehyde modification reaction to tRNAs.

FLUORESCENT G DERIVATIVES

We mentioned earlier the fluorescence of the natural Y bases,^{4-8,11,12} which are derivatives of guanosine, albeit with an extraneous methyl group at the 3-nitrogen. We have sought to provide synthetic G derivatives that would be fluorescent yet would not require additional alkylation at N-3 to produce the fluorescence. In addition, we have confined our search for fluorescence-inducing reagents to nonfluorescent molecules that would react selectively with guanosine and/or 7-methylguanosine under mild conditions in aqueous media, compatible to the fluorescent modification of G in RNAs, DNAs, nucleotides, coenzymes, etc.

Malondialdehyde reacts with DNA to form fluorescent products of unknown altered structure which emit at 460 nm upon excitation at 390 nm.^{114,115} When suitable controls were applied to the malondialdehyde reaction, we found that malondialdehyde alone produces products absorbing at 345 and 263 nm, with fluorescence emission at 455 nm, and exhibits the same new spots on cellulose tlc plates as one obtains when any of the following is present: adenosine, cytidine, guanosine,

Chemical reaction scheme showing the synthesis of a pyrazoloquinoline derivative. A substituted pyrazole (X-phenyl-pyrazole) reacts with a substituted pyrazole-4-carbonyl compound to form a fused pyrazoloquinoline system.

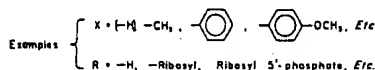
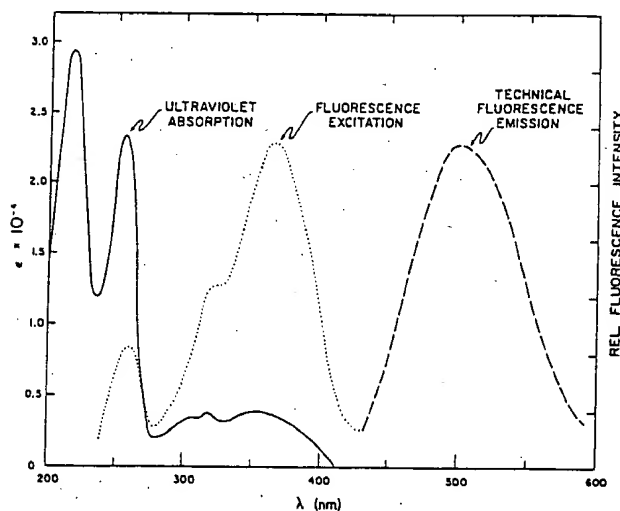
Cc1nc2c(ncn2C(=O)n1)c3ncn3 pH 6.8

FIGURE 2. Ultraviolet absorption, fluorescence excitation, and technical fluorescence emission spectra of 1,*N*²-(2-methylallylidene)guanine in aqueous solution buffered at pH 6.8.

Leonard & Tolman:

maxima, at pH 6.8 in aqueous long wavelength region of the that of the Y base. Upon excitation fluorescence emission of 1.1 (TABLE I). Other product 1, *N*²-(methylallylidene)guanosine

Compound	TECH
1,N ² -(2-methylallyl- idene)guanine (XXIII, R = H, X = CH ₃)	0.1
Disodium 1,N ² -(2-methyl- allylidene)guanosine 5'- monophosphate (dihydrate)	0.1

* Fluorescence emission spectra w
excitation maximum.

† Excitation spectra were measured.

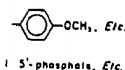
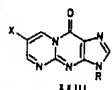
† Intensity of the emission maximum

characterized in the same manner as the interesting and useful region of the spectrum. The characterization of guanosine derivatives have also employed methylmalonate spray reagent for this purpose. The derivatives corresponding to XXIII are not expected to be G coenzyme surrogates with the exception of the 1-NH and 2-NH groups, which will be dependent, *inter alia*, on the demands that the 1-NH and 2-NH groups make. The rate of reaction is also being investigated. The rate of reaction in aqueous solution (ca. 0.7 nsec), of these derivatives, encourages us to investigate their structural modification in order to obtain the desired products. Finally, we are also in the process of studying, hopefully, site-specific, reactions at the 1-NH and 2-NH positions of the important modified nucleotides. As a caveat, reagents like chloroacetaldehyde should be handled carefully since they are

ACK

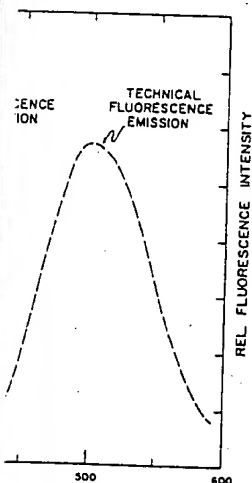
We wish to thank our colleagues who contributed to the researches described, Leslie H. Kirkegaard, Robert C. McPrem D. Sattsangi. We owe special thanks to D. Spencer for their contributions in

he fluorescence produced by malondialdehyde at 455 nm, providing a superrennity¹⁵ for adenine and guanine. The less, and if it is served up in water-soluble entities should result from G itself (XXII, X = H), Dr. Robert C. in obtaining well-characterized proguanosine, and the corresponding alondialdehydes (XXII, X ≠ H), as



on. The reaction is carried out in excess of excess substituted malondialdehyde for 24 hours but is advisably followed by XXII, R = H) and methylmalondialdehyde (XXIII, R = H), and nmr (base peak at 201(M⁺)), and nmr. The compound exhibited ultraviolet

pH 6.8



excitation, and technical fluorescence emission in aqueous solution buffered at pH 6.8.

maxima, at pH 6.8 in aqueous solution, at 355, 319, 309(sh), 256, and 218 nm. The long wavelength region of the absorption spectrum (FIGURE 2) bears resemblance to that of the Y base. Upon excitation at the long wavelength absorption maximum, the fluorescence emission of 1,N²-(2-methylallylidene)guanine occurs at 548 nm (TABLE I). Other products, for example, the disodium salt of 1,N²-(methylallylidene)guanosine 5'-monophosphate, as the dihydrate, have been

TABLE I
TECHNICAL FLUORESCENCE DATA

Compound	pH	Fluorescence Emission (nm)*			Fluorescence Excitation (nm)†
		λ_{max}	$\lambda_{+1/2}$	$\lambda_{-1/2}$	
1,N ² -(2-methylallylidene)guanine (XXIII, R = H, X = CH ₃)	6.8	500	548	460	360,325(sh),260
	0.1 N HCl	500	550	450	345,318(sh),295 (sh),250
Disodium 1,N ² -(2-methylallylidene)guanosine 5'-monophosphate (dihydrate)	10.1‡	470	538	425	360,325(sh),255
	6.8	500	550	455	360,325(sh),260
	0.1 N HCl	500	550	455	360,315(sh),250
	10.1‡	500	552	455	360,325(sh),260

* Fluorescence emission spectra were measured with excitation at the longest wavelength excitation maximum.

† Excitation spectra were measured by holding the fluorescence emission at 500 nm.

‡ Intensity of the emission maximum decreases with time, suggesting instability at this pH.

characterized in the same manner. The fluorescence emission for these lies in an interesting and useful region of the spectrum and thus should prove effective for the characterization of guanosine derivatives having an unsubstituted 2-NH₂ group. We have also employed methylmalondialdehyde (XXII, X = CH₃) qualitatively as a spray reagent for this purpose. The substituted 1,N²-(allylidene)guanylic acid derivatives corresponding to XXIII are currently under investigation as possible fluorescent G coenzyme surrogates with various enzymes. Whether they show activity or not will be dependent, *inter alia*, on whether the structural requirement for activity demands that the 1-NH and 2-NH₂ be free. Selectivity of reaction with G's in tRNA is also being investigated. The rather short fluorescent lifetimes thus far observed in these derivatives, encourages us to experiment further with substitution and structural modification in order to obtain improved G reagents.

Finally, we are also in the process of developing fluorescent base-specific, and, hopefully, site-specific, reactions and spray reagents for the other major nucleosides and the important modified nucleosides such as those found in the tRNAs. As a caveat, reagents like chloroacetaldehyde that react readily with nucleic acid bases must be handled carefully since they are potential mutagens.^{11,7,118}

ACKNOWLEDGMENTS

We wish to thank our colleagues at the University of Illinois who have contributed to the researches described, especially Jorge R. Barrio, John C. Greenfield, Leslie H. Kirkegaard, Robert C. Moschel, Mineo Saneyoshi, John A. Secrist, III, and Prem D. Sattsangi. We owe special thanks to Prof. Gregorio Weber and Dr. Richard D. Spencer for their contributions in theory, apparatus, and guidance.

REFERENCES

1. STRYER, L. 1968. *Science* 162: 526-533.
2. UDENFRIEND, S. 1962. *Fluorescence Assay in Biology and Medicine*. Vol. 1. Academic Press. New York, N.Y.
3. UDENFRIEND, S. 1969. *Fluorescence Assay in Biology and Medicine*. Vol. 2. Academic Press. New York, N.Y.
4. CANTOR, C. R. & T. TAO. 1971. *Procedures in Nucleic Acid Research*. G. L. Cantoni & D. R. Davies, Eds. Vol. 2. Harper & Row. New York, New York.
5. FURUTACHI, N., M. FIMANUZU, K. NAKANISHI, D. GRUNBERGER & I. B. WEINSTEIN. 1970. *J. Amer. Chem. Soc.* 92: 7617-7619.
6. KASAI, H., M. GOTO, S. TAKEMURA, T. GOTO & S. MATSUMARA. 1971. *Tetrahedron Lett.* 2725-2728.
7. BLOBSTEIN, S. H., D. GRUNBERGER, I. B. WEINSTEIN & K. NAKANISHI. 1973. *Biochemistry* 12: 188-193.
8. KREISHMAN, G. P., J. P. MILLER, P. DEA, Z. HUSSAIN, L. A. WILSON & M. P. SCHWEIZER. 1974. *Biochem. Biophys. Res. Commun.* 58: 27-34.
9. LANG, E., F. POCHON & A. M. MICHELSON. 1968. *Biochim. Biophys. Acta* 169: 338-349.
10. SCHEIT, K. H. 1970. *Biochim. Biophys. Acta* 209: 445-454.
11. SECRIST, J. A. III. 1972. Ph.D. Thesis. University of Illinois. Urbana, Ill.
12. EISINGER, J. & A. A. LAMOLA. 1971. *Methods Enzymol.* 21: 24-91.
13. EISINGER, J., B. FEUER & T. YAMANE. 1970. *Proc. Natl. Acad. Sci. USA* 65: 638-644.
14. BEARDSLEY, K., T. TAO & C. R. CANTOR. 1970. *Biochemistry* 9: 3524-3532.
15. THIEVE, R. & H. G. ZACHAU. 1970. *Biochim. Biophys. Acta* 217: 294-304.
16. MAELICKE, A., F. VON DER HAAR & F. CRAMER. 1973. *Biopolymers* 12: 27-43.
17. EISINGER, J., B. FEUER & T. YAMANE. 1971. *Nature [New Biol.]* 231: 126-128.
18. ROMER, R., D. REISNER & G. MAASS. 1970. *FEBS Lett.* 10: 352-357.
19. ZIMMERMAN, T. P., B. ROBISON & J. H. GIVVS. 1970. *Biochem. Biophys. Acta* 209: 151-160.
20. ROBISON, B. & T. P. ZIMMERMAN. 1971. *J. Biol. Chem.* 246: 110-117.
21. ROBISON, B. & T. P. ZIMMERMAN. 1971. *J. Biol. Chem.* 246: 4664-4670.
22. KRAUSS, G., R. ROMER, D. REISNER & G. MAASS. 1973. *FEBS Lett.* 30: 6-10.
23. TAO, T., J. H. NELSON & C. R. CANTOR. 1970. *Biochemistry* 9: 3514-3524.
24. YOSHIKAMI, D., G. KATZ, E. B. KELLER & B. S. DUDOCK. 1968. *Biochim. Biophys. Acta* 166: 714-717.
25. BEARDSLEY, K. & C. R. CANTOR. 1970. *Proc. Natl. Acad. Sci. USA* 65: 39-46.
26. WARD, D. C., E. REICH & L. STRYER. 1969. *J. Biol. Chem.* 244: 1228-1237.
27. WARD, D. C. & E. REICH. 1972. *J. Biol. Chem.* 247: 705-719.
28. GRUNBERGER, D., D. C. WARD & E. REICH. 1972. *J. Biol. Chem.* 247: 720-724.
29. WARD, D. C., A. GERAMI, E. REICH, G. ACS & L. ALTWERGER. 1969. *J. Biol. Chem.* 244: 3243-3250.
30. MAELICKE, A., M. SPRINZL, F. VON DER HAAR, T. A. KHWAJA & F. CRAMER. 1974. *Eur. J. Biochem.* 43: 617-625.
31. BRDAR, B. & E. REICH. 1972. *J. Biol. Chem.* 247: 725-730.
32. CHURCHICH, J. E. 1963. *Biochim. Biophys. Acta* 75: 274-276.
33. MILLAR, D. B. & R. F. STEINER. 1966. *Biochemistry* 5: 2289-2301.
34. MILLAR, D. B. & M. MACKENZIE. 1966. *Biochem. Biophys. Res. Commun.* 23: 724-729.
35. MILLAR, D. B. 1967. *Biochem. Biophys. Res. Commun.* 28: 70-75.
36. MILLAR, D. B. & M. MACKENZIE. 1967. *Biochemistry* 6: 2520-2526.
37. MILLAR, D. B. 1969. *Biochim. Biophys. Acta* 174: 32-42.
38. PACHMANN, U., E. CRONVALL, R. RIGLER, R. HIRSCH, W. WINTERMEYER & H. G. ZACHAU. 1973. *Eur. J. Biochem.* 39: 265-273.
39. LYNCH, D. C. & P. R. SCHIMMEL. 1974. *Biochemistry* 13: 1852-1861.
40. LYNCH, D. C. & P. R. SCHIMMEL. 1974. *Biochemistry* 13: 1841-1852.
41. YANG, C. H. & D. SÖLL. 1973. *Arch. Biochem. Biophys.* 155: 70-81.
42. WINTERMEYER, W. & H. G. ZACHAU. 1971. *FEBS Lett.* 18: 214-218.
43. WINTERMEYER, W. & H. G. ZACHAU. 1974. *Methods Enzymol.* 29: 667-673.
44. FRIEST, W., A. MAELICKE, M. SPRINZL & F. CRAMER. 1972. *Eur. J. Biochem.* 31: 215-220.
45. FINK, L. M., S. NISHIMURA & I. E.
46. PULKRABEK, P., D. GRUNBERGER
47. FUJIMURA, S., D. GRUNBERGER, C. 3629-3635.
48. SECRIST, J. A. III., J. R. BARRIO & I. 45: 1262-1270.
49. BARRIO, J. R. & N. J. LEONARD. 1973. *J. Biol. Chem.* 248: 1262-1270.
50. YANG, C. H. & D. SÖLL. 1973. *J. Biol. Chem.* 248: 1262-1270.
51. YANG, C. H. & D. SÖLL. 1974. *Biochemistry* 13: 1852-1861.
52. YANG, C. H. & D. SÖLL. 1974. *Proc. Natl. Acad. Sci. USA* 71: 1262-1270.
53. VELICK, S. F. 1961. *In Light and Life*. Hopkins Press. Baltimore, Md.
54. WEBER, G. 1950. *Biochem. J.* 47: 1-10.
55. SPENCER, R. D. & G. WEBER. 1972. *Enzymes*. A. Akesson & A. Ehrenl. 3: 1-10.
56. WEBER, G. 1958. *J. Chim. Phys.* 55: 1-10.
57. WEBER, G. 1957. *Nature* 180: 1409.
58. VELICK, S. F. 1958. *J. Biol. Chem.* 233: 1-10.
59. SCOTT, T. G., R. D. SPENCER, N. J. 687-695.
60. LAMBERT, R. F. 1967. Ph.D. Thesis.
61. KOCHETKOV, N. K., V. W. SHIBAIEV
62. BARRIO, J. R., J. A. SECRIST III & N. 46: 597-604.
63. SECRIST, J. A. III, J. R. BARRIO, N. 3499-3506.
64. KOCHETKOV, N. K., V. N. SHIBAIEV 100-103.
65. WANG, A. H.-J., L. G. DAMMANN, J. I. 1205-1213.
66. SHAW, G. & B. M. SMALLWOOD. 1973. *Tetrahedron Lett.* 14: 1205-1213.
67. YIP, Y. F. & K. C. TSOU. 1973. *Tetrahedron Lett.* 14: 1205-1213.
68. JONES, G. H., D. V. K. MURPHY, D. 53: 1338-1343.
69. CHHEDA, G. B., S. P. DUTTA, A. M. 433-436.
70. MEYER, R. B., Jr., D. A. SHUMAN, R. I. 16: 1319-1323.
71. ZBIRAL, E. & E. HUGL. 1972. *Tetrahedron Lett.* 13: 1327-1330.
72. KOCHETKOV, N. K., V. N. SHIBAIEV & 1327-1330.
73. EVANS, B. E. & R. V. WOLFENDEN. 1974. *Drug Evaluation*. communication.
74. GREENBERG, N. H. *Drug Evaluation*. communication.
75. SCHRAM, K. H. & L. B. TOWNSEND. 1974. *Drug Evaluation*. communication.
76. SPENCER, R. D., G. WEBER, G. L. TOI 45: 425-429 (1974).
77. MCCUBBIN, W. D., G. E. WILKICK & (50: 926-933.
78. DELUCA, M., N. J. LEONARD, B. J. GA: 70: 1664-1666.
79. SECRIST, J. A. III, J. R. BARRIO & N. 95: 961-962.
80. BARRIO, J. R., L. G. DAMMANN, L. H. K. 95: 961-962.
81. MOWERY, P. C. 1973. *Arch. Biochem. Biophys.* 155: 70-81.
82. MAHLEN, A. 1973. *Eur. J. Biochem.* 36: 1-10.
83. SHAHAK, Y., D. M. CHIPMAN & N. SH 36: 1-10.

- gy and Medicine. Vol. 1. Academic
- gy and Medicine. Vol. 2. Academic
- ic Acid Research. G. L. Cantoni &
rk, New York.
- UNBERGER & I. B. WEINSTEIN. 1970.
- ATSUMARA. 1971. Tetrahedron Lett.:
- : K. NAKANISHI. 1973. Biochemistry
- L. A. WILSON & M. P. SCHWEIZER.
- ochim. Biophys. Acta 169: 338-349.
- i-454.
- Illinois. Urbana, Ill.
- iol. 21: 24-91.
- itl. Acad. Sci. USA 65: 638-644.
- emistry 9: 3524-3532.
- s. Acta 217: 294-304.
3. Biopolymers 12: 27-43.
- [New Biol.] 231: 126-128.
- it. 10: 352-357.
- iochem. Biophys. Acta 209: 151-160.
- n. 246: 110-117.
- n. 246: 4664-4670.
73. F. Lett. 30: 6-10.
- emistry, J: 3514-3524.
- DOCK. 1968. Biochim. Biophys. Acta
- cad. Sci. USA 65: 39-46.
- Chem. 244: 1228-1237.
- 705-719.
- Biol. Chem. 247: 720-724.
- TWERGER. 1969. J. Biol. Chem. 244:
- KHWAJA & F. CRAMER. 1974. Eur. J.
- 5-730.
- 274-276.
- 5: 2289-2301.
- iophys. Res. Commun. 23: 724-729.
- un. 28: 70-75.
- y 6: 2520-2526.
- 2-42.
- RSCH, W. WINTERMEYER & H. G.
- y 13: 1852-1861.
- y 13: 1841-1852.
- phys. 155: 70-81.
- ett. 18: 214-218.
- s Enzymol. 29: 667-673.
- c. 1972. Eur. J. Biochem. 31: 215-220
45. FINK, L. M., S. NISHIMURA & I. B. WEINSTEIN. 1970. Biochemistry 9: 496-502.
46. PULKRABEK, P., D. GRUNBERGER & I. B. WEINSTEIN. 1974. Biochemistry 13: 2414-2419.
47. FUJIMURA, S., D. GRUNBERGER, G. CARVAJAL & I. B. WEINSTEIN. 1972. Biochemistry 11: 3629-3635.
48. SECRIST, J. A. III., J. R. BARRIO & N. J. LEONARD. 1971. Biochem. Biophys. Res. Commun. 45: 1262-1270.
49. BARRIO, J. R. & N. J. LEONARD. 1973. J. Amer. Chem. Soc. 95: 1323-1328.
50. YANG, C. H. & D. SÖLL. 1973. J. Biochem. (Tokyo) 73: 1243-1247.
51. YANG, C. H. & D. SÖLL. 1974. Biochemistry 17: 3615-3621.
52. YANG, C. H. & D. SÖLL. 1974. Proc. Natl. Acad. Sci. USA 71: 2838-2842.
53. VELICK, S. F. 1961. In Light and Life. W. D. McELROY and B. GLASS, Eds.: 108-143. Johns Hopkins Press. Baltimore, Md.
54. WEBER, G. 1950. Biochem. J. 47: 114-121.
55. SPENCER, R. D. & G. WEBER. 1972. In Structure and Function of Oxidation Reduction Enzymes. A. Akeson & A. Ehrenberg, Eds.: 393-399. Pergamon Press. New York, N.Y.
56. WEBER, G. 1958. J. Chim. Phys. 55: 878-886.
57. WEBER, G. 1957. Nature 180: 1409.
58. VELICK, S. F. 1958. J. Biol. Chem. 233: 1455-1467.
59. SCOTT, T. G., R. D. SPENCER, N. J. LEONARD & G. WEBER. 1970. J. Amer. Chem. Soc. 92: 687-695.
60. LAMBERT, R. F. 1967. Ph.D. Thesis. University of Illinois. Urbana, Ill.
61. KOCHETKOV, N. K., V. W. SHIBAEV & A. A. KOST. 1971. Tetrahedron Lett.: 1993-1996.
62. BARRIO, J. R., J. A. SECRIST III & N. J. LEONARD. 1972. Biochem. Biophys. Res. Commun. 46: 597-604.
63. SECRIST, J. A. III, J. R. BARRIO, N. J. LEONARD & G. WEBER. 1972. Biochemistry 11: 3499-3506.
64. KOCHETKOV, N. K., V. N. SHIBAEV & A. A. KOST. 1972. Dokl. Akad. Nauk. SSSR 205: 100-103.
65. WANG, A. H.-J., L. G. DAMMANN, J. R. BARRIO & I. C. PAUL. 1974. J. Amer. Chem. Soc. 96: 1205-1213.
66. SHAW, G. & B. M. SMALLWOOD. 1970. J. Chem. Soc. (C): 2206-2208.
67. YIP, Y. F. & K. C. TSOU. 1973. Tetrahedron Lett.: 3087-3090.
68. JONES, G. H., D. V. K. MURPHY, D. TEGG, R. GOLLING & J. G. MOFFATT. 1973. Biochem. Biophys. Res. Commun. 53: 1338-1343.
69. CHHEDA, G. B., S. P. DUTTA, A. MITTELMAN & L. BACZYNSKYJ. 1974. Tetrahedron Lett.: 433-436.
70. MEYER, R. B., Jr., D. A. SHUMAN, R. K. ROBINS, J. P. MILLER & L. N. SIMON. 1973. J. Med. Chem. 16: 1319-1323.
71. ZBIRAL, E. & E. HUGL. 1972. Tetrahedron Lett.: 439-444.
72. KOCHETKOV, N. K., V. N. SHIBAEV & A. A. KOST. 1973. Dokl. Akad. Nauk. SSSR C 213: 1327-1330.
73. EVANS, B. E. & R. V. WOLFENDEN. University of North Carolina. Private communication.
74. GREENBERG, N. H. Drug Evaluation Branch of the National Cancer Institute. Private communication.
75. SCHRAM, K. H. & L. B. TOWNSEND. 1974. Tetrahedron Lett.: 1345-1348.
76. SPENCER, R. D., G. WEBER, G. L. TOLMAN, J. R. BARRIO & N. J. LEONARD. 1974. Eur. J. Biochem. 45: 425-429 (1974).
77. MCCUBBIN, W. D., G. E. WILLOCK & C. M. KAY. 1973. Biochem. Biophys. Res. Commun. 50: 926-933.
78. DELUCA, M., N. J. LEONARD, B. J. GATES & W. D. McELROY. 1973. Proc. Natl. Acad. Sci. USA 70: 1664-1666.
79. SECRIST, J. A. III., J. R. BARRIO & N. J. LEONARD. 1972. Science 175: 646-647.
80. BARRIO, J. R., L. G. DAMMANN, L. H. KIRKEGAARD, R. L. SWITZER & N. J. LEONARD. 1973. J. Amer. Chem. Soc. 95: 961-962.
81. MOWERY, P. C. 1973. Arch. Biochem. Biophys. 159: 374-377.
82. MAHLEN, A. 1973. Eur. J. Biochem. 36: 342-346.
83. SHAHAK, Y., D. M. CHIPMAN & N. SHAVIT. 1973. FEBS Lett. 33: 293-296.

84. STEINER, R. F. 1972. *FEBS Lett.* 23: 139-141.
85. FELICIA, W. Y.-H., K. NATH & C.-W. WU. 1974. *Biochemistry* 13: 2567-2572.
86. LAWRENCE, F., D. J. SHIRE & J. WALKER. 1974. *Eur. J. Biochem.* 41: 73-81.
87. SECRIST, J. A. III, J. R. BARRIO, N. J. LEONARD, C. VILLAR-PALASI & A. G. GILMAN. 1972. *Science* 177: 279-280.
88. BARRIO, J. R., J. A. SECRIST III, Y. CHIEN, P. J. TAYLOR, J. L. ROBINSON & N. J. LEONARD. 1973. *FEBS Lett.* 29: 215-218.
89. WILICK, G. E., K. OIKAWA, W. D. MCCUBBIN & C. M. KAY. 1973. *Biochem. Biophys. Res. Commun.* 53: 923-928.
90. TONDRE, C. & G. G. HAMMES. 1973. *Biochim. Biophys. Acta* 314: 245-249.
91. ONISHI, H., E. OHTSUKA, M. IKEHARA & Y. TONOMURA. 1973. *J. Biochem. (Tokyo)* 74: 435-450.
92. CHIEN, Y. & G. WEBER. 1973. *Biochem. Biophys. Res. Commun.* 50: 538-543.
93. AVIGAD, G. & S. DAMLE. 1973. *Anal. Biochem.* 50: 321-323.
94. TRAMS, E. G. 1973. *J. Neurochem.* 21: 995-997.
95. LEONARD, N. J., J. R. BARRIO & J. A. SECRIST, III. 1972. *Biochim. Biophys. Acta* 269: 531-532.
96. JANIK, B., R. G. SOMMER, M. P. KOTICK, D. P. WILSON & R. J. ERICKSON. 1973. *Physiol. Chem. & Phys.* 5: 27-36.
97. LEHRACH, H. & K. H. SCHEIT. 1973. *Biochim. Biophys. Acta* 308: 28-34.
98. STEINER, R. F., W. KINNIE, A. LUNASIN & J. DELAC. 1973. *Biochim. Biophys. Acta* 294: 24-37.
99. LEE, C. H. & J. G. WETMUR. 1973. *Biochem. Biophys. Res. Commun.* 50: 879-885.
100. HERTZ, H. S. & H. G. ZACHAU. 1973. *Eur. J. Biochem.* 37: 203-213.
101. PACHMANN, U., E. CRONVALL, R. RIGLER, R. HIRSCH, W. WINTERMEYER & H. G. ZACHAU. 1973. *Eur. J. Biochem.* 39: 265-273.
102. ZACHAU, H. G. & H. S. HERTZ. 1974. *Eur. J. Biochem.* 44: 289-291.
103. BARRIO, J. R., J. A. SECRIST III. & N. J. LEONARD. 1972. *Proc. Natl. Acad. Sci. USA* 69: 2039-2042.
104. LEE, C.-Y. & J. EVERSE. 1973. *Arch. Biochem. Biophys.* 157: 83-90.
105. HARVEY, R. A. & DAMLE, S. 1972. *FEBS Lett.* 26: 341-343.
106. BARRIO, J. R., G. L. TOLMAN, N. J. LEONARD, R. D. SPENCER & G. WEBER. 1973. *Proc. Natl. Acad. Sci. USA* 70: 941-943.
107. TOLMAN, G. L., J. R. BARRIO & N. J. LEONARD. 1974. *Biochemistry* 13: 4869-4878.
108. WARSHAW, M. & I. TINOCO, Jr. 1966. *J. Mol. Biol.* 20: 29-38.
109. ARNOTT, S. 1971. *Prog. Biophys. Mol. Biol.* 22: 181-213.
110. BUGG, C. E., J. M. THOMAS, M. SUNDARALINGAM & S. T. RAO. 1971. *Biopolymers* 10: 175-219.
111. RUBIN, J., T. BRENNAN & M. SUNDARALINGAM. 1972. *Biochemistry* 11: 3112-3128.
112. SUSSMAN, J., N. C. SEEMAN, S.-H. KIM & H. BERMAN. 1972. *J. Mol. Biol.* 66: 403-421.
113. BRODYE, S. B., S. D. STELLMAN, B. HINGERTY & R. LANGRIDGE. 1974. *Biopolymers*. In press.
114. BROOKS, B. R. & O. L. KLAMERTH. 1968. *Eur. J. Biochem.* 5: 178-182.
115. REISS, U., A. L. TAPPEL & K. S. CHIO. 1972. *Biochem. Biophys. Chem. Commun.* 48: 921-926.
116. FRIHART, C. R. University of Illinois, Urbana, Ill. Unpublished.
117. RANNUG, U., A. JOHANSSON, C. RAMEL & C. A. WACHTMEISTER. 1974. *Ambio* 3: 194-197.
118. AMES, B. N. University of California, Berkeley, Calif. Private communication.

DISCUSSION OF THE PAPER

DR. CHHEDA: We have studied, in rats, the metabolic fate of ethenoadenosine labeled in the 2-position of the heterocycle. We did not find any significant radioactivity in transfer RNA, but approximately 80% of the administered radioactivity was excreted in the urine, 50% of that representing unchanged ethenoadenosine.

NUCLEOSIDES XCI. 5 OF THE ANTILEUKI β -D-ARABINOFURA

Jack JI

Memorial Slo
New Y

One of the most recent develop agent 1- β -D-arabinofuranosylcytosine that the 2,2'-anhydro derivative (3) experimental neoplasms in mice th effective when administered on do with ara-C.⁴ This is an important r complex and precise dosage schedu the inactivation of ara-C by enzym (2), a metabolite with no antitumor deamination,^{1,5} and studies on its n results from a slow, nonenzymic Anhydro-ara-C may therefore be cl.

A large number of analogs and patent and chemical literature. Amc the past several years at Sloan-Kett pound that is highly active against active than ara-C against cell-lines r to deamination by human-liver and ing ara-FU (5) unlike ara-U, is a c fourfold dose levels ara-FU shows a leukemia as does 5-fluoro-2'-deoxyu is akin to that of 5-fluorouracil, whe of ara-C.⁷⁻¹⁰ The 2,2'-anhydro de premise that slow hydrolysis wou form ara-FU. Anhydro-ara-FC mig each of which would exert its effect l tion of DNA polymerase or of thyn Anhydro-ara-FC has indeed prc mouse leukemias.¹¹ At approximat FC was more effective than ara-C, a single dose both when administere tion, its activity as a single dose i leukemia compares favorably wi chloroethyl)-1-nitrosourea. Anhydro an antileukemic agent.

Before we consider some aspects

* Supported in part by the National Ca can Cancer Society (grant CI-65N).

Immunofluorescent Demonstration of Double-Stranded RNA and Virus Antigen in RNA Virus-Infected Cells

I. N. GAVRILOVSKAYA, I. K. LAVROVA, M. K. VOROSHILOVA, M. P. CHUMAKOV, A. M. POVERENNY, AND V. K. PODGORODNICHENKO

The USSR AMS Institute of Poliomyelitis and Viral Encephalitides, Moscow, USSR and the USSR AMS Institute of Medical Radiology, Obninsk, Kaluga Region, USSR

Accepted July 24, 1974

The indirect immunofluorescence procedure has been used for demonstration of double-stranded RNA in cells infected with reovirus, poliomyelitis, and tick-borne encephalitis (TBE) viruses. Rabbit sera against poly(A)-poly(U) and poly(I)-poly(C) react specifically with double-stranded RNA. Double-stranded RNA is found in the cytoplasm of the cells infected with high multiplicities of poliomyelitis and tick-borne encephalitis viruses 3 hr postinoculation. In parallel, preparations were stained with sera against viral proteins. During one cycle of reproduction the dynamics of accumulation of double-stranded RNA and virus protein was synchronous both for poliomyelitis and tick-borne encephalitis viruses. When poliovirus-infected cells degenerated, the number of cells containing TBE virus double-stranded RNA decreased markedly while the proportion of cells containing virus protein remained high.

The high sensitivity and specificity of immunologic methods makes them useful for demonstration of nucleic acids in places where they are present in such low amounts that they are undetectable by other methods.

As has been demonstrated by Stollar *et al.* (1-4), animals immunized with MBSA¹—double-stranded synthetic polyribonucleotide complexes—develop antibody reacting specifically with double-stranded RNA. These data were confirmed in our study (5).

Recently, antisera for synthetic double-stranded polyribonucleotide complexes began to be used for demonstration of double-stranded RNA in cells infected with RNA viruses. For this purpose the double-

stranded RNA is either preextracted from the cells and then identified by means of the antiserum in an appropriate immunologic test or demonstrated in the cells by the direct or indirect immunofluorescence test (1, 2).

The present paper reports the use of the indirect immunofluorescence test with antibody against double-stranded polyribonucleotide complexes in order to determine the dynamics of accumulation of double-stranded RNA in the cytoplasm of the cells infected with poliomyelitis virus (three types), tick-borne encephalitis virus, and to demonstrate Reo virus. In parallel, the dynamics of virus protein accumulation was determined by immunofluorescence.

HeLa cells were grown on slides and infected with the prototype strains of poliomyelitis virus: type I (Mahoney), II (Neva), and III (Saukett). The multiplicity of infection was about 1000 PFU/cell. The virus yield reached maximum by 4 hr and was 1000-3000 PFU/cell.

¹Abbreviations used: MBSA, methylated bovine serum albumin; poly(A), polyadenylic acid; poly(U), polyuridylic acid; poly(AU), copolymer of riboadenylic acid and ribouridylic acid; poly(I), polyinosinic acid; poly(C), polycytidylic acid; poly(A)-poly(U), polyadenylic acid-polyuridylic acid complex; poly(I)-poly(C), polyinosinic acid-polycytidylic acid complex.

Hipr, Bars strains) was propagated in pig embryo kidney cells (SPEV). The input multiplicity was approximately 10 TCD₅₀/cell. The maximum accumulation of virus in the culture fluid was observed by 48 hr and the virus yield was 10-50 TCD₅₀/cell.

At intervals the slides were removed, fixed with cold acetone, and stained by the indirect immunofluorescence technique (6).

The sera from two rabbits immunized with MBSA-poly(A)-poly(U) complex (serum No. 21) and MBSA-poly(I)-poly(C) complex (serum No. 22) were used in the study in 1:16 dilutions. The immunization schedule was reported elsewhere (5, 7).

The sera against poliomyelitis and tick-borne encephalitis viruses were prepared by two to four inoculations of rabbits. Their titers were 1:1280 in neutralization tests for poliovirus of the three types and 1:1280 in hemagglutination-inhibition tests and 1:64 in immunodiffusion tests by the method of Ouchterlony for tick-borne encephalitis virus and were used in 1:8 dilution.

Fluorescein-conjugated donkey anti-rabbit gamma globulin was obtained from the Gamaleya Institute of Epidemiology and Microbiology and used in 1:8 dilution. Horse serum albumin conjugated with rhodamine sulfofluoride (8) was added as a counterstain.

The controls consisted of (a) noninfected cells; (b) cells fixed immediately after inoculation, (c) cells stained with rabbit serum collected before immunization, and (d) cells stained with a heterologous conjugate (against mouse globulins). All controls gave negative results.

The specificity of the sera against double-stranded polyribonucleotides was demonstrated in passive hemagglutination and in antibody neutralization tests (9). Thus, sera Nos. 21 and 22 reacted to titers 1:2560 and 1:5120, respectively, with erythrocytes loaded with poly(A)-poly(U) and did not react with erythrocytes loaded with poly(A), ribosomal RNA, single-stranded DNA. The passive hemagglutination test could be inhibited by preincubation with 0.3 μ g poly(A)-poly(U) or poly(I)-poly(C) and failed to be inhibited by preincubation

with poly(I)-poly(C), poly(A), poly(U), or ribosomal RNA used in 10- μ g amounts. The specificity of the test was confirmed by the data obtained by radioimmunoassay (Table 1). It will be seen in Table 1 that antibody for double-stranded polyribonucleotide complexes reacts only with double-stranded polyribonucleotides and not with single-stranded polyribonucleotides.

In green monkey kidney cells infected with reovirus I and stained with sera against double-stranded polyribonucleotides, fluorescence was observed at all stages of infection.

To test the specificity of fluorescence, 0.2 ml of undiluted serum No. 22 was incubated with 10 μ g poly(I)-poly(C) at 37°C for 1 hr followed by centrifugation at 10,000 rpm for 10 min. Serum was also incubated with 500 mg poly(A); 500 mg poly(U); 500 mg native thymus DNA; or phosphate buffer solution (control).

Subsequent staining of cells infected with Reo I and TBE (Sophylin) viruses showed that serum No. 22 after treatment

TABLE 1

RELATIVE ABILITIES OF NONLABELED DOUBLE-STRANDED AND SINGLE-STRANDED POLYRIBONUCLEOTIDES IN COMPETITION FOR ANTIBODY TO POLY(A)-POLY(U) (SERUM NO. 21) WITH ³H DOUBLE-STRANDED RNA*

Competing polyribonucleotide	(μ g)	Binding (cpm)
Poly(A)-poly(U)	5	53
Poly(A)	50	918
Poly(U)	50	958
Poly(AU)	50	923
—	50	962

* The incubation mixture consisted of 2.5 μ l undiluted test serum to which 0.1 ml SSC (0.15 M sodium chloride and 0.015 M sodium citrate) unlabeled polyribonucleotides (5 or 50 μ g) was added. After incubation at room temperature for 30 min, 1 μ g ³H-double-stranded RNA (approximately 1200 cpm) in 0.1 ml SSC was added. After incubation for 30 min at room temperature the mixture was passed through a membrane filter (Synpor 8, CSSR) at a flow rate of approximately 1 ml/min. The filter was washed two times with SSC, dried, and the nucleic acid-antibody complex was measured by determination of radioactivity on the filter in the Ansitron scintillation spectrometer. Similar results were obtained with serum No. 22.

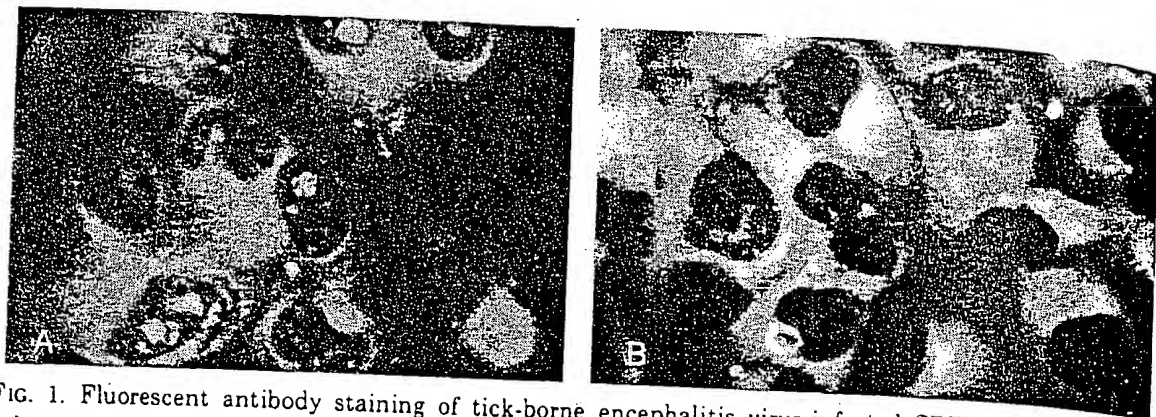


FIG. 1. Fluorescent antibody staining of tick-borne encephalitis virus-infected SPEV cells. The staining was done using rabbit anti-poly(A)-poly(U) (1%) serum No. 21 and fluorescein-labeled donkey anti-rabbit gamma globulin (1%). Magnification 90×5 . A. Six hours after infection. B. Twenty-four hours after infection.

with poly(I)-poly(C) lost its capacity to detect double-stranded RNA, whereas the control serum retained its reactivity completely.

The foregoing permits a conclusion that by means of the indirect immunofluorescence procedure we have demonstrated specific reaction of antibody against double-stranded polyribonucleotide complexes in the cytoplasm of the infected cells.

Specific fluorescence in the cytoplasm of the infected cells was detectable beginning at 3 hr after inoculation with tick-borne encephalitis virus and poliovirus. The character and localization of fluorescence were identical after infection of the cells with poliomyelitis or tick-borne encephalitis virus. At early stages of infection fluorescence was observed in the perinuclear zone (Fig. 1A), then it filled the cytoplasm in the form of large granules. At late stages fluorescence was diffuse throughout the cytoplasm. In some cells, fluorescence of nucleoli was also observed (Fig. 1B). Identical fluorescence was observed after staining with antiserum Nos. 21 and 22.

The dynamics of accumulation of double-stranded RNA and proteins of poliomyelitis and tick-borne encephalitis viruses in the cytoplasm of infected cells is shown in Fig. 2 which presents the results of one out of four analogous experiments. It will be seen in Fig. 2A that accumulation of double-stranded RNA and protein of poliomyelitis virus type 1 occurs simultaneously. The maximum number of fluorescent cells (70-76%) was observed 4 hr

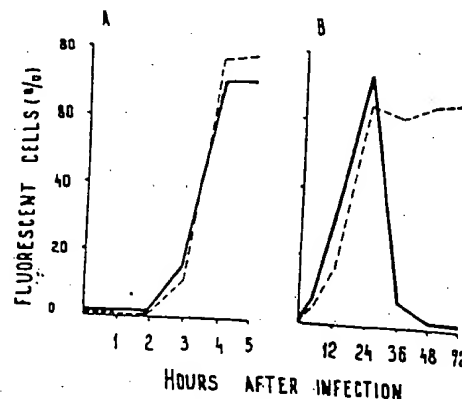


FIG. 2. The dynamics of accumulation of double-stranded RNA and protein of poliomyelitis virus type 1 (A) and tick-borne encephalitis virus (Sophy strain) (B) in the cytoplasm of the infected cells. The cells were grown on slides, fixed at different intervals postinfection, and half the slide was stained with a serum against double-stranded polyribonucleotides and the other half with antiserum for poliomyelitis or tick-borne encephalitis virus. Then both halves were stained with fluorescent anti-rabbit gamma globulin. At each half, 500 cells were counted and the percentage of fluorescent cells was calculated. — cells containing double-stranded RNA; - - - cells containing viral protein.

postinfection after which time the portion of cells containing viral protein and double-stranded RNA remained unchanged till the end of the virus reproduction cycle. Double-stranded RNA of poliomyelitis virus persisted in the cell until its complete destruction. Similar results were obtained with all three types of poliomyelitis virus.

The portion of the cells containing double-stranded RNA and protein of tick-borne encephalitis virus (Sophy strain) was similar during one reproduction cycle and up to 24 hr after infection.

quently the portion of cells containing double-stranded RNA decreased considerably and after 48 hr fluorescence was found in only 2-3% of cells, whereas viral protein was observed in the cytoplasm of 60% of cells at all intervals of the observation period. Similar results were obtained also with the Absettarov and Hipr strains.

Thus, during one reproduction cycle the dynamics of accumulation of double-stranded RNA and viral protein is synchronous with poliomyelitis and tick-borne encephalitis viruses. Then, at high multiplicities of infection with poliomyelitis virus the cells degenerate and with TBE virus the number of cells containing double-stranded RNA decreases.

This study has demonstrated that detection of double-stranded RNA by the immunofluorescence method is a reliable test both for entero- and arbovirus infection of cells and may be used for study of the virus-cell interaction.

ACKNOWLEDGMENT

We are grateful to Dr. G. A. Shirman for his comments and suggestions.

REFERENCES

1. STOLLAR, V., and STOLLAR, B. D., *Proc. Nat. Acad. Sci. USA* 65, 993-1000 (1970).
2. STOLLAR, B. D., and STOLLAR, V., *Virology* 42, 276-280 (1970).
3. SCHWARTZ, E. F., and STOLLAR, B. D., *Biochem. Biophys. Res. Commun.* 35, 115-120 (1969).
4. STOLLAR, B. D., *Science* 169, 609-611 (1970).
5. POVERENNY, A. M., PODGORODNICHENKO, V. K., and SAPRYGIN, D. V., *Immunochemistry* 10, 577-581 (1973).
6. COONS, A. H., In "General Cytochemical Methods" (J. F. Danielli, ed.), pp. 400-422, Academic Press, New York, 1958.
7. PLESCIA, O., BRAWN, W., and PALEZUK, N., *Proc. Nat. Acad. Sci. USA* 52, 279-285 (1964).
8. NOSKOV, F. S., BOLDYREV, V. K., GOLDIN, R. V., ERMAKOV, N. V., and VOLKOVA, L. A., *Vopr. Virusol.* 10, 613-614 (1965) (in Russian).
9. POVERENNY, A. M., and LEVI, M. T., *Biokhimiya* 29, 80-87 (1964) (in Russian).

staining
it gamma

f double
virus type
(Sophyia)
cells. The
intervals
d with a
nucleotides
yelitis or
lves were
globulin
percent
— cells
- - cells

portion
d dou-
anged
cycle.
yelitis
mplete
tain-
vi.
ig dou-
f tick-
strain)
n cycle

sh for

PEPTIDYL-tRNA WITH A FLUORESCENT LABEL: RIBOSOME SUBSTRATES IN PEPTIDE BOND FORMATION

M. K. KUKHANOVA, A. A. KRAYEVSKY, L. A. ALEXANDROVA,
S. A. STRELTSOV, AND B. P. GOTTIKH

Institute of Molecular Biology, U.S.S.R. Academy of Sciences, Moscow, U.S.S.R.

(Received 7 June, 1974)

I. INTRODUCTION

Fluorescent label introduced in ribosomes may help elucidate some questions of its functioning and structure. There are both direct and indirect methods of introduction of labels, fluorescing in the long-wave region, to ribosome. The former may be exemplified by reactions involving fluoresceinylisothiocyanate [1], the latter is based on modification of tRNA and its binding to ribosome. Among these modifications are substitution of some bases in tRNA by ethidium bromide or proflavin [2], addition of acryflavin and 9-hydrazino acrydine to the oxidized end of tRNA [3], addition of the dansyl and anthanoyl residues to the 5'-phosphate end of tRNA [4] and formation of a fluorescing component by irradiating tRNA^{Val} from *E. coli* with UV light [5].

This paper reports a method of introduction of fluorescent residues (dansyl, anthracene-2-sulphonyl and fluoresceinyl) which bind to the amino acid moiety of aminoacyl-tRNA. Such peptidyl-tRNA preserve the ability of being specifically bound to ribosomes in the presence of a template and of being peptide donors in ribosome in the reaction with pyromycin (Pu).

II. MATERIALS AND METHODS

Use was made of tRNA from *E. coli* containing 20% of the phenylalanine accepting fractions. [¹⁴C]Phenylalanine, specific activity 225 Ci mole⁻¹, was the product of UVVVR, Czechoslovakia; enzymatic aminoacylation was performed as described elsewhere [6]. Radioactivity was measured in an ABAC SL-30 scintillating spectrophotometer (Intertechnique). Dansyl (DNS) dipeptidyl-tRNA was prepared as previously described [7]. The starting anthracenylsulphochloride (ANT-Cl) was condensed with glycine similarly to a DNS-amino acid synthesis [8]; ANT-Gly (m.p. 198°) was then converted into non-crystalline ANT-Gly-OSu (where OSu is N-hydroxysuccinimide residue) [7]. This substance was condensed with [¹⁴C] Phe-tRNA into ANT-Gly-[¹⁴C]Phe-tRNA. Glycine was added to fluoresceinylisothiocyanate (Reachim, U.S.S.R.) and converted into fluoresceinylaminothiocarbonyl glycine, FLU-Gly [9], m.p. above 320°, which with HOSu and dicyclohexylcarbodiimide (30 min at 0° and 1 hr at 20°) was converted into FLU-Gly-OSu, which was then condensed without further purification with [¹⁴C]Phe-tRNA into FLU-Gly-[¹⁴C]Phe-tRNA according to Alexandrova *et al.* [7], but the reaction was only run for 2.5 hr. All the peptidyl-tRNAs were isolated and purified by means of chromatography on Sephadex G-25 as

TABLE I
Synthesis of peptidyl-tRNA (III) containing a fluorescent label

Taken				III Synthesized		Yield of III per II (%)
X in I	II, A ₂₆₀ units	II, radio- activity cpm × 10 ⁻³	A ₂₆₀ units	Radio- activity cpm × 10 ⁻³	Content of III in preparation calculated by radioactivity, %	
DNS-Gly-	4	216	2.55	151	100	70.0
DNS-Ala-	4	216	2.15	147	93	63.2
DNS-Val-	4	216	2.12	165	93	71.0
DNS-Phe-	4	216	2.05	169	90	70.4
ANT-Gly-	26.1	240	22.6	151	95	59.7
FLU-Gly-	22.0	163	20.5	112	94	64.5

^a Experiments were performed with Phe-tRNA in total tRNA preparations.

TABLE II
Poly U-stimulated binding of X-[¹⁴C]Phe-tRNA (III) by ribosomes

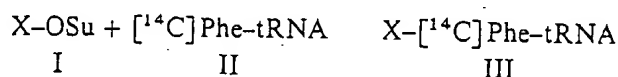
No.	X in III	Quantity of III cpm	Binding, cpm			Stimulation of binding	
			+ poly U	- poly U	+ poly A	+ poly U - poly A	+ poly U + poly A
1	DNS-Gly	6500	3400	2500	-	2.3	-
2	DNS-Gly	6500	2400	1230	-	1.95	-
3	DNS-Ala-	11330	6360	3630	-	1.75	-
3	DNS-Ala-	12700	6960	3630	-	1.9	-
4	DNS-Val-	10000	4050	2680	-	1.5	-
4	DNS-Val-	11600	5600	3900	1660	1.45	3.4
5	DNS-Val-	10000	3480	2340	-	1.53	-
6	DNS-Phe-	10560	5000	5100	-	1	-
6	DNS-Phe-	7500	7400	6910	4880	1.07	1.5
7	DNS-Phe	10560	3435	2730	-	1.27	-
8	ANT-Gly-	12720	4110	-	2740	-	1.5
9	Ac-	11150	5200	865	-	6.0	-
10	Ac-	5040	3020	565	-	5.3	-

Experimental conditions: The incubation mixture contained 3 A₂₆₀ units ribosomes, 0.8 A₂₆₀ units poly U (or 2.0 A₂₆₀ units poly A), 5 μmol Tris-HCl pH 7.5, 1 μmol MgCl₂, 16 μmol NH₄Cl in 0.1 ml. Time of incubation was 20 min at 30°. In cases No. 2, 5, 7 and 10 the reaction mixture was placed in a column 1 × 19 cm with Sephadex G-100 (Buffer: 0.01 M Tris-HCl, 0.01 M MgCl₂, pH 7.6). After separation optical density at 260 nm and radioactivity were determined. Radioactivity (measured by means of dioxane scintillator) was calculated for 3 A₂₆₀ units of solution. In other cases the reaction was stopped by adding 2 ml the same cold buffer, the mixture was filtered through VUFS nitro-cellulose filters (Chemapol, Czechoslovakia) and washed with 25 ml of the buffer. The radioactivity was determined in a toluence scintillator.

described earlier [7]. The quantity of non-acylated Phe-tRNA in the preparations synthesized was determined by hydrolyzing of aliquots of substances in 0.25 N NaOH (20°, 2 hr); the hydrolysate was then chromatographed on FN-16 paper (D.D.R.) using BuOH-AcOH-water (78:5:17 v/v). The radioactive zone was determined. In this system phenylalanine has an R_f value of 0.38 and its fluorescing peptide derivatives - 0.8-0.9. The purity of all intermediate low molecular weight compounds was determined by TLC on silica gel or silicic acid.

III. RESULTS AND DISCUSSION

The scheme of synthesis of peptidyl-tRNA containing a fluorescent label is based on condensation of N-hydroxysuccinimide esters of N-acylated amino acids and [14 C]Phe-tRNA by the method of Lapidot [10]:



where X is DNS-Gly-, DNS-Ala-, DNS-Val-, DNS-Phe-, ANT-Gly- or FLU-Gly-. The yield and the content of peptidyl-tRNA in the preparations obtained are listed in Table I, which shows that the amount of Phe-tRNA in them does not exceed 10%.

All the preparations of peptidyl-tRNA (III) were investigated in a cell-free system with ribosomes from *E. coli* MRE-600 in the presence of poly U and referred to the same system being bound without the template or with poly A. As is seen from Table II, all preparations of type III

TABLE III

Donor activity of peptidyl-tRNA X- $[^{14}\text{C}]$ Phe-tRNA (III)

NN	III	Content, $\text{cpm} \times 10^{-3}$	Extracted into ethylacetate, $\text{cpm} \times 10^{-3}$				Donor activity %
			+ Pu	- Pu	+ Pu	- Pu	
1	Ac	13.3	11.4	3.0	8.4		63
2	DNS-Gly	5.8	4.0	2.1	1.9		33
3	DNS-Ala	8.4	6.85	2.9	3.95		47
4	DNS-Val	7.5	4.95	2.2	2.75		55.5
5	DNS-Phe	8.7	7.9	4.5	3.4		37
6	ANT-Gly	12.7	3.55	2.1	1.45		11
7	ANT-Gry	12.7	4.53	3.4	2.13		9
8 ^a	FLU-Gly	17.6	11.98	6.7	5.28		30
9 ^a	FLU-Gly ^b	17.6	9.76	6.4	3.35		19
10 ^a	FLU-Gly ^c	17.6	8.53	5.5	2.83		16

^a In experiments No. 8-10 radioactivity was determined by the precipitate of III in 10% trichloroacetic acid.

^{b, c} The content of ethanol in incubation mixture - 39 and 45% respectively.

Experimental conditions: The incubation mixture contained in 0.1 ml 3 A₂₆₀ units ribosomes, 38 mM KCl, 2 mM MgCl₂ and 5.7 mM Tris-HCl, pH 7.8 at 20°. The reaction was initiated by adding 50 μ l ethanol, performed for 1 hr and stopped by adding 0.1 ml 0.01 M Tris-HCl buffer pH 7.0 and 3 ml ethylacetate. After extraction the radioactivity was determined in 2 ml of organic phase in 15 ml of the solution containing toluene scintillator and methylcellosolve 1:1 v/v.

displayed an ability of binding with ribosomes, but, unlike AcPhe-tRNA used as a reference, their binding in the absence of the template is anomalously high. However, it decreases with lower hydrophobicity of the peptide on the one hand, and with poly A as a template on the other. These facts prompt one to the suggestion that an increase in the hydrophobicity of the peptide sharply increases the ability of peptidyl-tRNA to bind to the peptidyltransferase centre (PTC) of ribosomes even if no template is present. This is supported by the data on the more efficient binding between the donor site of PTC and acylaminoacyl oligonucleotides as compared to aminoacylnucleotide [11] and peptidyl-tRNA as compared with aminoacyl-tRNA [12]. There are reasons for believing that the peptide moiety of peptidyl-tRNA in ribosomes has a hydrophobic environment.

Table III shows the results of determination of the activity of III as peptide donors in a templateless system containing 31% of ethanol; Pu was used as peptide acceptor. All the peptidyl-tRNA synthesized (III) were able to serve as peptide donors, and in the case of DNS-derivatives the peptide-donating activity is sufficiently high. These data show that ribosomes are capable of binding with peptidyl-tRNA in the PTC donor site with rather bulky, and highly hydrophobic groups. A study of the peptide-donating activity of III in the ribosome-poly U system showed this ability not to be very high. One may think that this phenomenon is due to the ability of III to occupy simultaneously the donor and the acceptor sites of PTC even with 10 mmoles of Mg^{2+} , which precludes the reaction with Pu [13].

Summarizing, one may say that peptidyl-tRNA having a fluorescent label of sufficient size and hydrophobicity in the peptide moiety of the molecule may be specifically bound to ribosomes and may act as peptide donors in the reaction with Pu. It should be added that the ability of peptidyl-tRNA to become attached to the PTC of ribosomes increases as does hydrophobicity and is rather high even if the template is absent.

REFERENCES

1. Huang, K. and Cantor, C. R., *J. Mol. Biol.* 67, 265 (1972).
2. Wintermeyer, W. and Zachau, H. G., *FEBS Letters* 18, 214 (1971).
3. Churchrich, J. E., *Biochim. Biophys. Acta* 75, 274 (1963).
4. Yang, C. H. and Söll, D., *Arch. Biochem Biophys.* 155, 70 (1973).
5. Favre, A. and Yaniv, M., *Fed. Eur. Biochem. Soc. Lett.* 17, 236 (1971).
6. Lewin, J. C. and Nirenberg, M., *J. Mol. Biol.* 34, 467 (1968).
7. Alexandrova, L. A., Kukhanova, M. K., Krayevsky, A. A., Treboganov, A. D., and Gottikh, B. P., *Izv. Akad. Nauk SSSR, Ser. Biologich.* 426 (1973).
8. Hartley, B. S., *Biochem. J.* 119, 805 (1970).
9. Kawachi, H., Turimura, K., Maeto, H., and Ishida, N., *J. Biochemistry* 66, 783 (1969).
10. Lapidot, Y., de Groot, N., Weiss, M., Peled, R., and Wolman, Y., *Biochem. Biophys. Acta* 138, 241 (1967).
11. Celma, M. L., Monro, R. E., and Vazquez, D., *FEBS Letters* 13, 247 (1971).
12. Watanabe, S., *J. Mol. Biol.* 67, 443 (1972).
13. de Groot, N., Panet, A., and Lapidot, Y., *Israel J. Chem.* 10, 775 (1972).

- Monod, J., Wyman, J., and Changeux, J. P. (1965), *J. Mol. Biol.* 12, 88.
- Pearlmutter, A. F., and Stuehr, J. (1968), *J. Amer. Chem. Soc.* 90, 858.
- Pörschke, D., and Eigen, M. (1971), *J. Mol. Biol.* 62, 361.
- Reeves, R. H., Cantor, C. R., and Chambers, R. W. (1970), *Biochemistry* 9, 3993.
- Rialdi, G., Levy, J., and Biltonen, R. (1972), *Biochemistry* 11, 2472.
- Robison, B., and Zimmerman, T. P. (1971a), *J. Biol. Chem.* 246, 110.
- Robison, B., and Zimmerman, T. P. (1971b), *J. Biol. Chem.* 246, 4664.
- Rossi-Fanelli, A., Antonini, E., and Caputo, A. (1964), *Advan. Protein Chem.* 19, 74.
- Rossotti, F. J. C., and Rossotti, H. (1961), *The Determination of Stability Constants*, New York, N. Y., McGraw-Hill.
- Saneyoshi, M., Harada, F., and Nishimura, S. (1969), *Biochim. Biophys. Acta* 190, 264.
- Schofield, P., Joffman, B. M., and Rich, A. (1970), *Biochemistry* 9, 2525.
- Schreier, A. A. (1973), Ph.D. Thesis, M.I.T.
- Schreier, A. A., and Schimmel, P. R. (1972), *Biochemistry* 11, 1582.
- Schreier, A. A., and Schimmel, P. R. (1974), *J. Mol. Biol.* (in press).
- Takahashi, K. (1961), *J. Biochem. (Tokyo)* 49, 1.
- Tao, T., Nelson, J. H., and Cantor, C. R. (1970), *Biochemistry* 9, 3514.
- Ward, D. C., Reich, E., and Stryer, L. (1969), *J. Biol. Chem.* 244, 1228.
- Willick, G. E., and Kay, C. M. (1971), *Biochemistry* 10, 2216.
- Yang, S. K., Söll, D. G., and Crothers, D. M. (1972), *Biochemistry* 11, 2311.
- Yarus, M., and Barrell, B. G. (1971), *Biochem. Biophys. Res. Commun.* 43, 729.
- Yarus, M., and Berg, P. (1969), *J. Mol. Biol.* 42, 171.
- Yarus, M., and Rashbaum, S. (1972), *Biochemistry* 11, 2043.

Effects of Abnormal Base Ionizations on Mg^{2+} Binding to Transfer Ribonucleic Acid as Studied by a Fluorescent Probe†

Dennis C. Lynch‡ and Paul R. Schimmel*

ABSTRACT: The naphthoxyl probe attached to the 3'-end of isoleucyl-tRNA^{Ile} (see Lynch, D. C., and Schimmel, P. R. (1974), *Biochemistry* 13, 1841) has been used to study the pH dependence of Mg^{2+} binding at "cooperative" sites. The apparent Mg^{2+} affinity is strongly pH dependent; e.g., it is ca. tenfold and 100-fold weaker at pH 6 and pH 4.7, respectively, than at pH 7.5. This effect is due to abnormally high base pK's in the "aberrant" structure(s) formed in low salt, Mg^{2+} -free solutions. The emission of the probe is sensitive to the ionization of one of these sites, probably a cytidylic acid moiety near the 3'-end. Addition of sufficient Mg^{2+} sharply lowers the abnormal pK's to more typical values. The kinetics of Mg^{2+} addition at pH 6 appears to follow essentially the

same mechanism as at pH 7.5—two slow unimolecular changes coupled to rapid Mg^{2+} binding steps. However, the Mg^{2+} -induced structural changes are slower, have somewhat higher activation energies, and are thermodynamically less favored at pH 6. These effects apparently arise from the greater stability of aberrant form(s) brought about by base protonations, and they largely account for the weaker apparent binding of Mg^{2+} observed by fluorescence at pH 6 as opposed to pH 7.5. Ultraviolet absorption data corroborate many of the findings. Preliminary results with tRNA^{Ala} (*Escherichia coli*) labeled with the probe are similar to those obtained with tRNA^{Ile}, thus suggesting that the results obtained may be rather general.

In the preceding paper (Lynch and Schimmel, 1974), it was shown that the fluorescence emission of a naphthoxyl group attached to the 3'-end of tRNA^{Ile} is sensitive to the binding of Mg^{2+} to "interacting" or "cooperative" sites on the nucleic acid. Two slow unimolecular structural changes occur as Mg^{2+} is bound to these sites; these changes have large activation energies and are probably due to the breakdown of aberrant structures formed in the absence of Mg^{2+} (see Cole *et al.*,

1972). Since the structural changes induced by Mg^{2+} binding are thermodynamically favorable, they serve to increase the apparent strength of binding of Mg^{2+} . This accounts for the high affinity of Mg^{2+} binding to these sites.

At pH 6 we were surprised to learn that the binding of Mg^{2+} observed by fluorescence is significantly weaker than that observed at pH 7.5 (Lynch and Schimmel, 1974), even though there are no obvious base or phosphodiester ionizations in this pH range. The results presented below demonstrate that abnormal pK's (on bases) are present on tRNA and that protonation of these sites leads to an increased stabilization of the "aberrant" structure(s) formed in low salt. Addition of Mg^{2+} sharply lowers the abnormal pK's and encourages proper folding of the tRNA, although the folding process itself is somewhat slower and goes with higher activation energies at the more acid pH (pH 6) than at pH 7.5. The decreased thermodynamic preference for the Mg^{2+} -induced

† From the Departments of Biology and Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139. Received October 23, 1973. This work was supported by grant no. GM-15539 from the National Institutes of Health. Material for this manuscript was taken from the Ph.D. thesis of Dennis C. Lynch (Massachusetts Institute of Technology, 1973).

‡ Present address: Division of Biology, California Institute of Technology, Pasadena, Calif. 91109. Predoctoral Fellow of the National Institutes of Health, 1970-1973.

(1970), ²Bio- structural changes at pH 6 later accounts for the weaker apparent binding at this pH as opposed to pH 7.5.

Biochemistry Materials and Methods

Mo (in Many of the details of the preparation of materials, treatment of data, instrumentation, and etc., are given in the preceding paper (Lynch and Schimmel, 1974). Additional details pertinent to the present paper are given below.

Biochemistry Derivatized isoleucyl-tRNA^{Ile} in which the 5'-phosphate is removed was prepared by incubating IV¹ for 1 hr at 53° in 0.05 M Tris-0.1 mM MgCl₂ (pH 7.5) with ca. 1 μg of bacterial alkaline phosphatase (Worthington BAPF grade) per nmole of tRNA. These conditions, which are slightly milder than usual phosphatase conditions, were chosen in order to minimize hydrolysis of the label. Under these conditions, <15% of the label was lost. Phosphatase was removed *via* phenol extraction and an aliquot of the tRNA was digested for 5 hr at 37° in 0.01 M (NH₄)₂CO₃ (pH 8) with ca. 2 μg of RNase A (Worthington) per nmole of tRNA. This digest and a control not treated with phosphatase were applied to separate 20 × 20 cm cellulose thin-layer plates (E. Merck) and chromatographed in two dimensions (first dimension: 1-propanol-concentrated ammonia-water, 55:10:35 by volume; second dimension: isobutyric acid-concentrated ammonia-water, 66:1:33 by volume; U. L. RajBhandary and M. Simsek, personal communication; see also Saneyoshi *et al.* (1969)). The resultant patterns were examined under an ultraviolet lamp and found to be identical except that a slow moving oligonucleotide spot present in the control was absent in the phosphatase treated material. We attribute the missing spot to the 5'-terminal fragment pApGpGpCp which would be converted to ApGpGpCp by successful phosphatase removal of the tRNA 5'-phosphate. As there is another expected RNase A fragment of this identity (see Yarus and Barrell, 1971), a new spot is not expected in the phosphate treated pattern. Thus the chromatograms provide fair, although not rigorous, evidence that the 5'-phosphate was removed.

Polarization of fluorescence was measured with a Farrand Optical Company Mark I spectrofluorometer employing films supplied by the manufacturer. The measured values were corrected for apparent polarization introduced by the monochromator by the method of Azumi and McGlynn (1962; see also, Chen and Bowman (1965)). Fluorescence stopped-flow experiments were performed with a Durrum-Gibson stopped-flow equipped with a 75-W xenon lamp (General Electric). The apparatus has a dead time of less than 5 msec. (We gratefully acknowledge the use of this instrument at the Peter Bent Brigham Hospital, in the laboratory of Dr. B. Vallee. The helpful assistance of Dr. D. Auld is also acknowledged.) Fluorescence lifetimes were measured with the single photon counting system designed by Ortec in the laboratory of Dr. Renata Cathou at Tufts University Medical Center (see Lynch (1973), for additional details). The permission granted by Dr. Cathou to use the instrument, and the extensive help given by Dr. James Bunting are gratefully acknowledged.

The pH measurements made in fluorescence pH titrations were performed on thermostated samples not containing tRNA. The pH was measured directly with a Radiometer pH meter equipped with a GK2021C electrode; titrations were generally reproducible to within ±0.03 pH unit. The samples

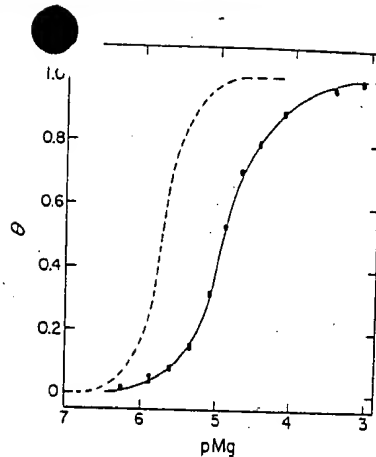


FIGURE 1: Relative change in fluorescence (θ) vs. pMg. Solid curve with points applies to data at pH 6.0, 10 mM Na⁺, 1 mM EDTA, 20 mM cacodylate, and Cl⁻ counterion. Dashed curve applies to data at pH 7.5, 10 mM Na⁺.

containing tRNA which were used for optical measurements were then titrated in the same way, but without directly measuring the pH. This procedure proved to be more efficient and also necessary because of fluorescence contamination introduced by the electrode. Fluorescence and uv titration were on occasion checked for reversibility. These titrations were generally found to be reversible over most of the pH range studied. Difficulty was sometimes encountered at acid pH values which promote incipient precipitation of tRNA.

EDTA was used to buffer Mg²⁺ concentrations as was done in the preceding study (Lynch and Schimmel, 1974). The stability constant of the Mg²⁺-EDTA complex increases with increasing pH (see Laitinen, 1960). Fortunately, this pH dependence is in the same direction as the pH dependence of Mg²⁺ binding to tRNA. This allowed the use of EDTA at several different pH values and concentrations to buffer Mg²⁺ in different concentration ranges.

Organic liquids used in fluorescence experiments were distilled when they contained obvious optical impurities.

Results and Treatment of Data

pH Dependence of Mg²⁺ Binding. Fluorescence Mg²⁺ titration of the derivatized tRNA at pH 6 gave strikingly different results than those obtained at pH 7.5. Figure 1 gives a plot of the fractional change in fluorescence θ vs. pMg at pH 6. For comparison, the results obtained at pH 7.5 are shown by a dashed line. It is clear from this figure that the Mg²⁺ binding observed by fluorescence is substantially weaker and less cooperative at pH 6.0 than at pH 7.5. This result is somewhat surprising since *a priori* it is not immediately apparent that there are any groups on tRNA which ionize in this region, except for the 5'-terminal phosphate. Any involvement of this group was eliminated, however, by the finding that the Mg²⁺ binding followed by fluorescence was not altered by apparent removal of this group with bacterial alkaline phosphatase.

The pH dependence of the Mg²⁺ binding was further pursued by performing fluorescence titrations at pH 4.7. The data obtained at the various pH values are tabulated in Table I in terms of the apparent dissociation constant K_{app} and the empirical Hill coefficient n . These parameters were obtained as described in the preceding paper (Lynch and Schimmel, 1974). In addition, some data on Na⁺ titrations are given also.

These data show a remarkable pH dependence of the Mg²⁺ affinity for tRNA. The affinity changes somewhat less than an

¹ Abbreviation used is: A_{λ} , the absorbance at wavelength λ of a solution in a 1-cm path-length cell. Structures I-IV are defined in Lynch and Schimmel (1974).

cular changes
r, f g²⁺
ewh. higher
less favored
greater sta-
protonations
nt binding of
ed to pH 7.5
the findings
coli) labeled
ith tRNA^{Ile}
ather general

Mg²⁺ binding
increase the
ounts for the

ie binding of
weaker than
, 1974), even
iester ioniza-
elow demon-
on tRNA and
sed stabiliza-
w salt. Addi-
and cour-
ldii process
er activation
7.5. The de-
Mg²⁺-induced

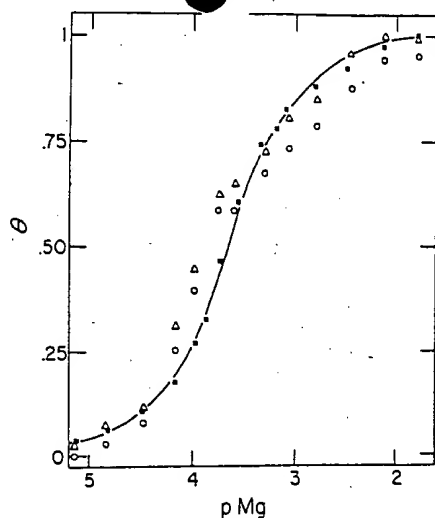


FIGURE 2: Plot of θ vs. pMg at pH 4.7, 25° with a buffer of 70 mM Na^+ , 0.1 M acetate, 10 mM EDTA, and Cl^- counterion: (■) θ for fluorescence; (O) θ for A_{260} ; (Δ) θ for A_{280} . Curve is drawn for fluorescence points.

order of magnitude for each pH unit. Furthermore, the cooperativity is most pronounced at pH 7.5 and is significantly less at the other pH values. The binding of Na^+ is also dependent on pH, although less markedly than Mg^{2+} . However, at all pH values Na^+ exerts relatively little influence on the apparent Mg^{2+} dissociation constant although it does depress the cooperativity.

At this point it is worth asking whether the remarkable pH effects are due to general effects of hydrogen ion on tRNA structure or if they might rather be due to a highly localized phenomenon sensed by the fluorescence probe. To answer this question, the small uv absorbance changes accompanying Mg^{2+} binding were also studied at each of the pH values. Figure 2 gives results obtained at pH 4.7 where θ (=fractional change in absorbance or fluorescence) vs. pMg is given. The points for fluorescence (■) and absorbance (A_{260} = O; A_{280} = Δ) fall about the same curve. An approximately similar correlation was found at pH 6.0. This indicates that the emission and absorbance changes occur in the same general region

TABLE I: pH-Dependence of Mg^{2+} and Na^+ Binding at 25°

pH	[Na^+] (mM)	pK_{app}	n
Mg^{2+} Titrations			
7.5 ^a	10	5.73	2.30
	37	5.79	1.52
6.0	10 ^b	4.84	1.26
	45 ^c	4.74	0.99
4.7	10 ^d	3.9 ^f	
	70 ^e	3.66	1.14
	100 ^e	3.70	1.06
Na^+ Titrations			
7.5		1.52	2.10
6.0		1.33	1.8
4.7		0.82	1.76

^a See Lynch and Schimmel (1974) for details. ^b 20 mM cacodylate, 1 mM EDTA, and Cl^- counterion. ^c 100 mM cacodylate, 1 mM EDTA, and Cl^- counterion. ^d 10 mM acetate, 1 mM EDTA, and Cl^- counterion. ^e 100 mM acetate, 10 mM EDTA, and Cl^- counterion. ^f Curve is very unsymmetric.

and that the pH induced effects are doubtless associated with the overall tRNA structure and not just a localized area.

pH Dependence of Fluorescence. The magnitudes of the emission changes associated with the Mg^{2+} binding discussed above are dependent on the pH. This indicates, of course, that the probe's emission is sensitive to hydrogen ion as well as metal ions. This is clearly seen in Figure 3a where the relative fluorescence at 350 nm is plotted vs. pH for two different conditions. The upper curve was obtained in 10 mM Mg^{2+} ; the lower curve was obtained in Mg^{2+} -free solutions containing 10 mM Na^+ . It is clear that under both conditions the emission is strongly pH dependent and appears to follow a simple titration curve. The magnitude of the emission changes and the apparent midpoints of the titration curves are very different, however. In both cases, the high pH plateau is achieved by pH 7.5, so that the Mg^{2+} titrations discussed in

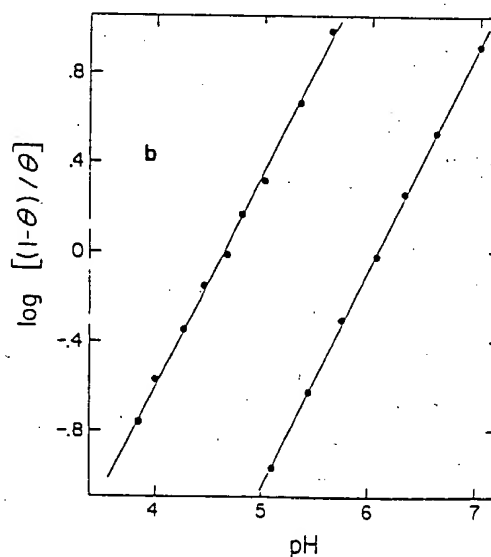
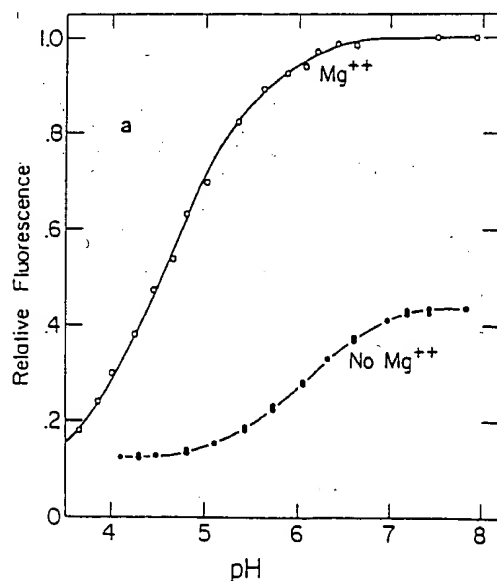


FIGURE 3: (a) Relative fluorescence emission vs. pH. The upper curve was obtained in 10 mM Mg^{2+} and the lower curve in 10 mM Na^+ . Details of experimental procedure are given in Table II. Points on the upper curve are averages of three experiments; those on the lower curve are from two experiments with scatter as shown (where there is only one point, observed values coincided). (b) Plot of $\log [(1 - \theta)/\theta]$ vs. pH for data derived from curves in Figure 3a.

TABLE II: Fluorescence pH Titrations of Labeled tRNA at 25°.

Cation (concn, mM)	Method	pK_H	n	Quench- ing ^c
Na ⁺ (10)	a	6.04 ± 0.03	1.01 ± 0.02	0.30
Na ⁺ (0.55 M)	b	4.57 ± 0.06	0.95 ± 0.04	0.11
Mg ²⁺ (10)	c	4.63 ± 0.05	0.99 ± 0.04	0.12
Mn ²⁺ (10)	d	4.64 ± 0.04	0.95 ± 0.03	0.19
Spermidine ³⁺ (1)	b	4.74 ± 0.05	1.01 ± 0.03	0.16

^a Buffer contained 3 mM EDTA, 10 mM Na⁺, and Cl⁻ counterion. Titration was from high pH to low pH with 0.1 and 1.0 M acetic acid. ^b Buffer contained 5 mM EDTA, 5 mM acetate, 10 mM NaCl, and 10 mM cacodylate, titrated from low pH to high pH with 0.1 M NaOH. ^c Buffer contained 10 mM cacodylate, 5 mM acetate, and 10 mM NaCl, titrated from low pH to high pH with 0.1 M NaOH. ^d Buffer contained 10 mM cacodylate and 10 mM NaCl; titration with 0.1 and 1.0 M acetic acid was from high pH to low pH. ^e Ratio of emission at low pH plateau to that at high pH plateau.

the preceding paper (Lynch and Schimmel, 1974) monitor the transition from one plateau to the other. There is clearly a low pH plateau on the low salt curve, and the high Mg²⁺ curve is apparently heading toward one. (Experiments below pH 3.5 could not be carried out because the tRNA precipitates at acid pH.)

The curves in Figure 3a were replotted as $\log [(1 - \theta)/\theta]$ versus pH according to the simple ligand binding scheme of the preceding paper (Lynch and Schimmel, 1974). The results are given in Figure 3b which shows that the data are very linear over the range $0.1 < \theta < 0.9$ and conform well to a single pK. Similar titrations were performed in the presence of large amounts of Mn²⁺ and spermidine as well. Table II summarizes the hydrogen ion pK_H and n values obtained. In every instance the data fit that for a single site with the cation stabilized form of the tRNA having its pK_H shifted about 1.3–1.4 units below that of the low salt form. The fact that the data fit that for a single pK under a variety of conditions indicates that the probe is probably monitoring just one ionization site on the tRNA, although the changes in Mg²⁺ affinity with pH may be brought about by ionizations at several sites.

What is the identity of these ionization sites? Although the pH dependence of tRNA structure has previously not been well studied, DNA and several synthetic polyribonucleotide systems have been well characterized with respect to pH dependent behavior. For example, strongly salt dependent pK_H values have been observed for protonations of the bases in DNA; in general, the lower the salt concentration, the higher the pK_H. Values between pK_H = 4.0 and 6.1 for deoxycytidylic acid residues, and between pK_H = 3.5 and 5.4 for deoxyadenylic acid residues in DNA have been reported (Cavalieri and Stone, 1955; Jordan, *et al.*, 1956; Cox and Peacocke, 1957; see Jordan, 1960, for an extensive review), whereas the monomers have approximately salt independent pK_H values of 4.2 and 3.6, respectively (Cavalieri and Stone, 1955). Protonation of the adenine bases in poly(A) leads to the formation of a double helix in which each adenine forms three hydrogen bonds (Rich *et al.*, 1961). The protonation occurs about pK_H = 5.9 in 0.1 M K⁺ and is "severely depressed" by 2 mM Ca²⁺ (Beers and Steiner, 1957; Steiner and Beers, 1959). Poly(C) can form a double helical, triple hydrogen bonded structure which exhibits pK_H's in solution of 5.7 and 3.0,

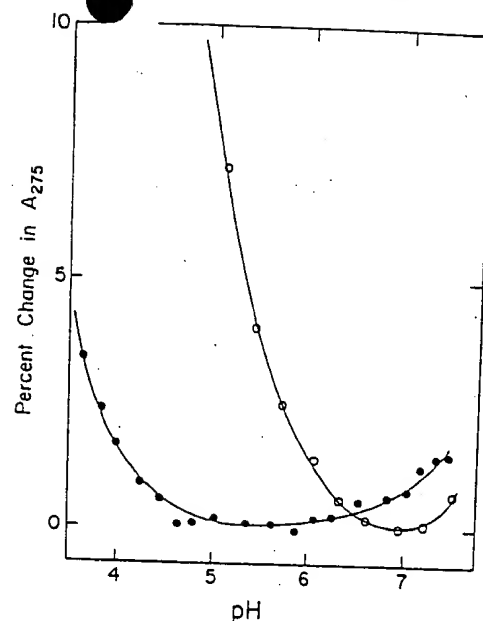


FIGURE 4: Per cent change in A_{275} vs. pH at 25°. Buffers are as for corresponding experiments in Figure 3a: (●) data obtained in the presence of 10 mM Mg²⁺; (○) data obtained in the presence of 10 mM Na⁺, no Mg²⁺. Details as for corresponding fluorescence titrations given in Table II.

compared with a pK_H of 4.2 for cytidylic acid (Langridge and Rich, 1963; Hartman and Rich, 1965). Each protonation corresponds to the addition of one proton per base pair. The abnormal pK_H values are interpreted as being a result of the first proton binding more readily (pK_H = 5.7) to allow formation of a particular hydrogen bond and subsequent helix formation, and the second proton binding weakly (pK_H = 3.0) because its presence destroys the helix.

In each of these examples, protonation at the elevated pK_H leads to the formation of additional structure in the polynucleotide. Therefore, it is plausible that protonation of specific groups on tRNA leads to additional structure, although not necessarily the same kinds of structures observed in cases mentioned above.

The ultraviolet absorption of unacylated tRNA¹¹⁶ was studied as a function of pH in order to assess further the pH dependent structural effects. Since A and C residues are likely to cause a pH effect, particular attention was directed to measurements at 275 nm where the protonation of C has its largest change (protonation of A causes almost no change in its spectrum (Cavalieri and Stone, 1955; Hartman and Rich, 1965)). Figure 4 shows the pH titration monitored at 275 nm of unacylated tRNA¹¹⁶ in 10 mM Mg²⁺ and in 10 mM Na⁺. The changes are rather small. The data definitely show, however, that the uv absorption at 275 nm is pH dependent. The titration curves do not appear to have a simple structure, presumably because the uv absorption change is made up of the overlapping contributions of many bases. However, the relative positions of the titration curves are markedly shifted by Mg²⁺ in a way which qualitatively resembles the effects followed by emission changes (Figure 3a). It seems likely from the uv absorption data that more than one C is being protonated in the region where the probe shows a pH dependence of its fluorescence. Although no specific data were obtained, it is reasonable to suspect that one or more A residues is also protonated in this region.

As mentioned above, the fluorescence data suggest that the probe is sensitive to only one of these "abnormal" ionizations. To check on the effects of nearby ionizations on the naph-

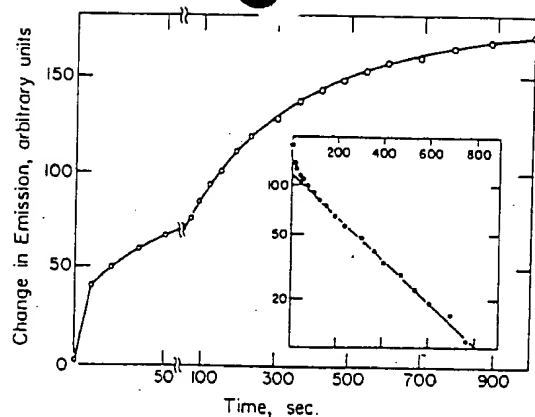


FIGURE 5: Change in fluorescence emission intensity with time following the addition of Mg^{2+} at pH 6.0, 25° with a buffer of 20 mM cacodylate, 1 mM EDTA, 10 mM Na^+ , and Cl^- counterion. Addition of Mg^{2+} resulted in a final free Mg^{2+} concentration of 6.3×10^{-4} M. The inset gives a semilogarithmic plot of the final signal minus the current signal vs. time.

thoxyl group's fluorescence, the pH dependence of the emission of the parent compound I and the fragments II and III was studied. All of these molecules display pH-dependent emission changes which follow a single pK_H . These titrations were different in two major respects from that of the label attached to intact tRNA, however. First, the quenching observed as the pH is lowered is significantly less than that found with intact tRNA (Lynch, 1973). Second, the titrations of I, II, and III are independent of Mg^{2+} which markedly contrasts with the results shown in Figure 3a for IV. In the case of I a $pK_H = 2.4$ was found. The pK_H apparently corresponds to ionization of the carboxyl group (phenoxycetic acid has a carboxyl $pK_H = 3.15$ (Hayes and Branch, 1943)). The observed fluorescence level and pH dependence of I in the pH 3.5–8 range are not changed if unmodified tRNA is added to the solution in fourfold greater concentration than is normally used in titrations of IV. This indicates that the fluorescence of the naphthoxyl moiety is not greatly influenced by the tRNA if they are not covalently bonded together.

A $pK_H = 3.8$ was observed for II. This pK_H closely corresponds to a pK_H of adenosine (Alberty *et al.*, 1951). This result suggests that the fluorescence of the naphthoxyl group is able to monitor protonations in its vicinity.

In the case of III, almost all of the signal change conforms to a smooth titration curve centered at pH 4.8; there was slight (<5% of the initial signal) additional quenching below pH 3.5 which was not studied. The pK_H value of 4.8 is about 1.2 units above that for the adenine base of AMP (Alberty, *et al.*, 1951) and 0.6 unit above that for the cytosine base of CMP (Hartman and Rich, 1965). Thus, it seems likely that a C rather than an A is responsible for the changes in fluorescence seen in III. If a cytidine(s) is responsible, the results indicate that the probe is sensitive to a protonation at a non-adjacent base in the primary structure.

The salt independent $pK_H = 4.8$ for III is very close to that observed in the high salt form of IV, which raises the possibility that the same group is causing the fluorescence changes in III and in IV. Of the four C residues in III, the two closest to the probe are not base paired in the tRNA cloverleaf structure. Either of them are plausible candidates for the locus of the protonation causing the fluorescence changes in IV.

Kinetic Studies at pH 6. The kinetic studies of Mg^{2+} binding reported in the preceding paper (Lynch and Schimmel, 1974) were done at pH 7.5 where the emission exhibits no pH dependence. To investigate further the mechanism of coupling

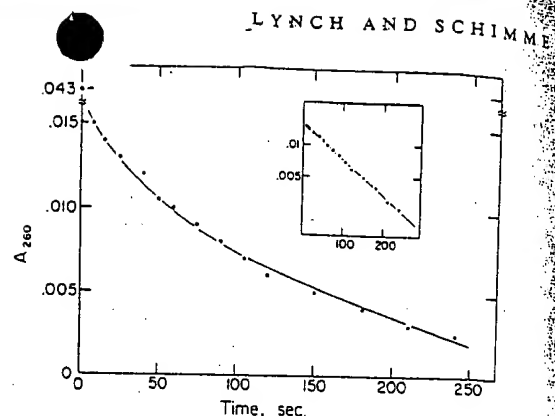


FIGURE 6: Change in A_{260} with time following the addition of Mg^{2+} pH 6.0, 25° with buffer as in Figure 5. Inset is ΔA_{260} on logarithmic scale. Addition resulted in a free $[Mg^{2+}] = 9.5 \times 10^{-5}$ M.

of the "abnormal" hydrogen ion equilibria with the cooperative binding of Mg^{2+} , kinetic studies were carried out at pH 6. At this pH (in 10 mM Na^+), the cooperativity index n is reduced about twofold and the apparent Mg^{2+} dissociation constant almost tenfold from the values observed at pH 7.5 (see Table I).

Figure 5 gives a plot of the time course of the fluorescence increase ΔF following Mg^{2+} addition at 25°. The kinetics clearly appear to involve at least three phases—a rapid jump followed by two slower phases. The inset in the figure gives $\log \Delta F$ vs. time. When the longer time portion of the semilogarithmic plot is subtracted from the total signal, the early time portion also yields a straight line when the data are replotted. However, unlike the case at pH 7.5, when the straight lines are extrapolated back to zero time, they do not account for all of the intensity change that occurs. This is a consequence of the rapid initial jump in fluorescence.

Similar rates for Mg^{2+} induced optical density changes were observed by monitoring the absorbance of unacylated tRNA^{11c} at 260 nm (see Figure 6). Although the changes are quite small (~5%), the time course may be resolved into two straight line sections on a semilogarithmic plot with the same rates as observed in the fluorescence experiments. The changes in optical density are observed with derivatized tRNA as well as the unacylated species. Since the uv changes are in general agreement with the fluorescence results, and since the same rates are observed for unacylated as well as derivatized tRNA^{11c}, it is concluded that at pH 6, as at pH 7.5, the probe is monitoring, but not influencing, a general tRNA conformational change.

The linearity of the semilogarithmic plots indicates that first-order, or pseudo-first-order, kinetic processes are causing the fluorescence changes. However, since the two observed processes do not account for all of the signal change, one (or more) additional rapid process is necessary to describe the observed overall change ΔF to give

$$\Delta F = \Delta F' + A_1 e^{-\lambda_1 t} + A_2 e^{-\lambda_2 t} \quad (1)$$

where $\lambda_1 > \lambda_2$, $\Delta F'$ is the rapid initial change in fluorescence that occurs on Mg^{2+} addition, A_1 and A_2 are amplitude parameters, and λ_1 and λ_2 are time constants. The parameters λ_1 and λ_2 are plotted as functions of the Mg^{2+} concentration (at 10 mM Na^+) in Figures 7a and b. Each exhibits a hyperbolic Mg^{2+} dependence. The lines in the figures are theoretical curves which are derived below. This behavior of λ_1 and λ_2 implies that these two rate processes represent slow unimolecular changes coupled to rapid bimolecular step(s). Thus, the slower time portions of the kinetics are qualitatively similar at pH 6 and pH 7.5.

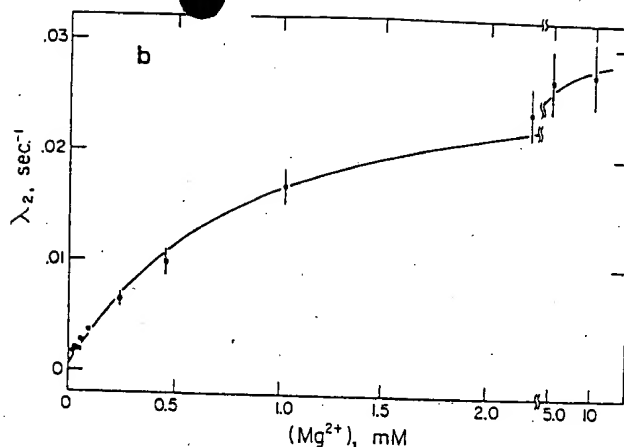
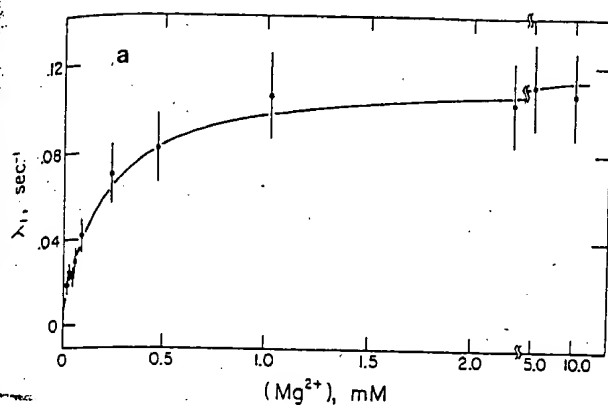


FIGURE 7: (a) Plot of λ_1 vs. $[Mg^{2+}]$ at pH 6.0, 25°. Points are averages of several experiments, the error bars are $\pm 20\%$. All experiments were done in 10 mM Na^+ , 20 mM cacodylate, 1 mM EDTA, and Cl^- counterion. The line is a theoretical curve calculated as described in the text with the parameters listed in Table III. (b) Plot of λ_2 vs. $[Mg^{2+}]$; the error bars are $\pm 10\%$. See legend to Figure 7a for further details.

In order to resolve the rate of the initial jump $\Delta F'$, fluorescence stopped-flow experiments were performed. It was observed that for Mg^{2+} additions giving Mg^{2+} concentrations of 10^{-3} and 10^{-4} M, the portion of the signal change corresponding to $\Delta F'$ was completely over within the dead time of the instrument ($\sim 5 \times 10^{-3}$ sec). This indicates that any bimolecular Mg^{2+} binding step(s) involved in generating $\Delta F'$ must have a rate constant(s) greater than $ca. 2 \times 10^6$ $M^{-1} sec^{-1}$. This is of the order seen for reaction between Mg^{2+} and ADP in a temperature-jump study (see Eigen and Hammes, 1960).

Despite the inability to measure a rate for $\Delta F'$, valuable information may be obtained from the Mg^{2+} dependence of its magnitude. Figure 8a gives a plot of θ_j vs. pMg where θ_j is the fractional change (of its maximal change) of $\Delta F'$, and Figure 8b gives $\log [(1 - \theta_j)/\theta_j]$ vs. pMg. These data yield $n = 1.0$ and $pK_{app} = 3.6$ suggesting that the $\Delta F'$ phase of the fluorescence change might be due to binding a single Mg^{2+} . A comparison of the high pMg plateau level of $\Delta F'$, $\Delta F'_{max}$, with the lower titration curve in Figure 3a shows that $\Delta F'_{max}$ is of the same size as expected for deprotonation of IV at pH 6. That the rapidity of the ionization effects was presumably associated with $\Delta F'$ was confirmed by the finding that the emission changes accompanying a pH jump in the absence of Mg^{2+} are too rapid to follow by manual techniques. These facts lead to the speculation that the rapid portion of the signal change is due to a deprotonation of IV resulting from an initial Mg^{2+} association. This postulation also explains why no rapid phase is observed at pH 7.5, since at that pH all of IV is deprotonated even in the absence of Mg^{2+} .

Kinetic Mechanism and Analysis. In spite of the marked differences in the equilibrium binding curves between pH 6.0 and 7.5, the kinetic data are quite similar. In each case two slow processes are observed, although the $Mg^{2+} \rightarrow \infty$ rates at pH 6 are about a factor of 3 lower than the equivalent rates at pH 7.5. All of the λ vs. $[Mg^{2+}]$ plots show hyperbolic dependence on Mg^{2+} concentration, although the midpoints of the curves at pH 6 fall at somewhat lower Mg^{2+} concentrations. The only qualitative difference in the two sets of observations is the appearance of the rapid phase ($\Delta F'$) at pH 6, and this difference is reasonably accounted for in terms of the protonation sensed by the probe. In light of these similarities, it is not unreasonable to suppose that a very similar mechanism is operating at pH 6 as at pH 7.5.

In order to extend to pH 6 the mechanism derived for pH 7.5, it is first necessary to reconsider the cause of $\Delta F'$. It has

been pointed out that $\Delta F'$ is probably caused by a deprotonation step of the type



where the $RH \rightleftharpoons R$ equilibrium refers to the ionization monitored by the probe. Since the rapid fluorescence change upon Mg^{2+} addition obviously precedes either of the unimolecular processes, it is logical to have eq 2 as the first step in the mech-

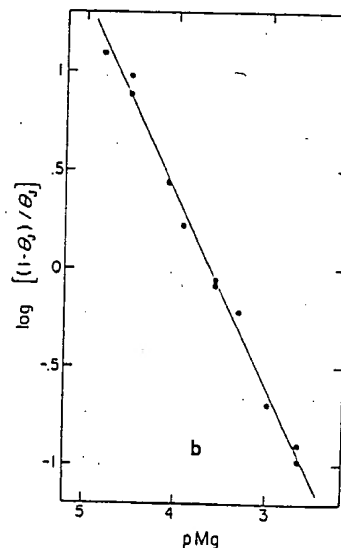
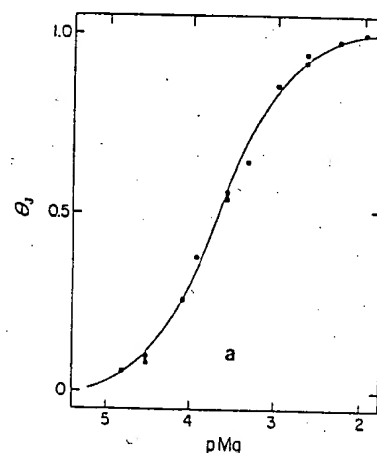


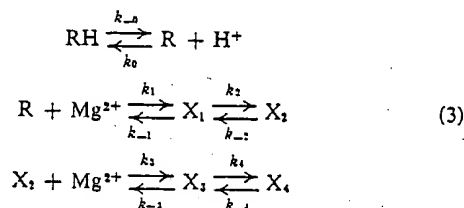
FIGURE 8: (a) Plot of θ_j vs. pMg, at pH 6.0, 25° with a buffer containing 10 mM Na^+ , 20 mM cacodylate, 1 mM EDTA, and Cl^- counterion. See text for details. (b) Plot of $\log [(1 - \theta_j)/\theta_j]$ vs. pMg. Data are derived from the curve in Figure 8a. See text for details.

TABLE III: Kinetic Parameters at pH 6.0, 25°.^a

Rate Constant (sec ⁻¹)		Equilibrium Constant (M)	
k_2	0.115	K_1	1.1×10^{-4}
k_{-2}	0.005	K_3	8.0×10^{-4}
k_4	0.030	$K_H = K_0$	$10^{-6.0}$
k_{-4}	0.0005		

^a All kinetic parameters were derived from data obtained at pH 6.0, 25° with solutions containing 1 mM EDTA, 20 mM cacodylate, 10 mM Na⁺, and Cl⁻ counterion.

anism. This will happen if Mg²⁺ preferentially binds to R and thus promotes dissociation of HR. Therefore, the overall scheme may be written as



It is again assumed that bimolecular steps in the mechanism are rapid compared to the $\text{X}_1 \rightleftharpoons \text{X}_2$ and $\text{X}_3 \rightleftharpoons \text{X}_4$ steps. Above ca. pH 7, eq 3 reduces to the mechanism studied at pH 7.5 (Lynch and Schimmel, 1974) since dissociation of RH to R is complete at that pH. Other mechanisms may be eliminated, as pointed out elsewhere (Lynch, 1973; Lynch and Schimmel, 1974).

The rate equations for the above mechanism may be derived in a manner analogous to that described in the preceding paper (Lynch and Schimmel, 1974). Some details are given in Appendix I. The solution of these equations gives expressions for λ_1 and λ_2 which may be used to extract equilibrium and kinetic parameters for the mechanism in the same manner as described in Lynch and Schimmel (1974). Results of this analysis are tabulated in Table III (where the equilibrium constant $K_i = k_{-i}/k_i$). The curves in Figures 7a and b were calculated on the basis of these parameters. It is seen that agreement of calculated with observed behavior is very good.

Two additional sets of data were tested for their compliance to the proposed mechanism. The first was the θ vs. pMg plot in Figure 1. Relative fluorescences were assigned to each of the species in the mechanism based on the amplitudes of three kinetic phases. An expression was derived for θ vs. $[\text{Mg}^{2+}]$ by using these relative fluorescence assignments together with the parameters in Table III. Appendix II gives additional details. The calculated values of the parameters characterizing the titration curve are $n = 1.32$, $pK_{\text{app}} = 4.73$; the observed values of these parameters are $n = 1.26$, $pK_{\text{app}} = 4.84$.

The second additional item of data tested against the mechanism was the amplitude $\Delta F'$ of the initial jump phase of the kinetics. According to our interpretation, this arises from deprotonation of RH caused by binding of Mg²⁺ to R. This involves the first two steps of the mechanism and the entire fluorescence change comes from the $\text{RH} \rightarrow \text{R} + \text{X}_1$ conversion since the emissions of R and X₁ are the same. We can therefore use the θ_1 vs. pMg plot (Figures 8a and b) to calculate K_1 . This calculation is given in Appendix II. The value so calculated is 1.2×10^{-4} M. The value obtained from the kinetic data (the λ_1 vs. $[\text{Mg}^{2+}]$ plot in Figure 7a) is 1.1×10^{-4} M (see Table III). It is clear that all available data conform extremely well to the proposed mechanism.

TABLE IV: Comparison of Equilibrium Constants at pH 7.5 and pH 6.0.

	pH 7.5 20°, 15 mM Na ⁺	pH 6.0 25°, 10 mM Na ⁺
K_{app} , M	2.3×10^{-6}	1.4×10^{-6}
n	1.8	1.3
K_1 , M	4.2×10^{-4}	2.2×10^{-4} ^a
K_2	1.0×10^{-2}	4.4×10^{-2}
K_3 , M	17×10^{-4}	8.0×10^{-4}
K_4	1.0×10^{-3}	17×10^{-3}
K_5 , M	8×10^{-6}	
K_{I} , ^b M	4×10^{-6}	1×10^{-5}
K_{II} , ^b M	2×10^{-6}	1×10^{-5}

^a $K_{\text{app}} = [\text{Mg}^{2+}]_{1/2, \lambda_1} = K_1/(1 + [\text{H}^+]/K_H)$. $[\text{Mg}^{2+}]_{1/2, \lambda_1}$ is the midpoint of the τ_1 vs. $[\text{Mg}^{2+}]$ plot. ^b $K_{\text{I}} = K_1/(1 + 1/K_2)$, $K_{\text{II}} = K_3/(1 + 1/K_4)$.

It is of considerable interest to compare the numerical values of the various equilibrium constants obtained at pH 6 with those at pH 7.5. Such a comparison should permit identification of the step(s) responsible for the tenfold drop in Mg²⁺ binding affinity and the large decrease in apparent cooperativity observed at pH 6. The conditions for the two sets of kinetic experiments were slightly different: the pH 6 data were obtained at 25°, 10 mM Na⁺, while data at pH 7.5 were obtained at 20°, 15 mM Na⁺. The use of identical temperatures and Na⁺ concentrations would further accentuate the differences between the data at pH 6 and pH 7.5, as may be seen by the greater cooperativity and Mg²⁺ affinity evident in the Mg²⁺ titration at pH 7.5, 10 mM Na⁺, 25° as opposed to one at pH 7.5, 15 mM Na⁺, 20° (see Lynch and Schimmel, 1974).

The comparative data are listed in Table IV; the constants K_1 and K_{II} are the apparent overall binding constants for each Mg²⁺ ion in the mechanism and are defined in the table legend. All of the parameters vary somewhat between the two pH values with the Mg²⁺ association constants K_1 and K_3 being stronger at pH 6. However, each of the isomerization constants K_2 and K_4 decreases in going from pH 7.5 to pH 6, with the most outstanding change coming in the $\text{X}_3 \rightleftharpoons \text{X}_4$ isomerization. The net effect of these changes is to make weaker the overall association of Mg²⁺ ions monitored by the probe.

It is also evident why the apparent cooperativity decreases at pH 6. The apparent dissociation constants change their relative values between pH 7.5 and pH 6; at pH 7.5, $K_{\text{II}} < K_1$ but at pH 6, $K_{\text{II}} = K_1$. The weakening of K_{II} relative to K_1 has the effect of spreading the fluorescence change over a larger range of Mg²⁺ values, thereby lowering the apparent cooperativity. In addition, the Mg²⁺ induced conversion of RH to R gives a contribution to the overall fluorescence change at pH 6 which is not present at pH 7.5. This gives greater weight, at pH 6, to the fluorescence change associated with the first Mg²⁺ binding, which in turn manifests itself in a greater first power Mg²⁺ component in the log $(1 - \theta)/\theta$ vs. pMg plots.

Finally, the temperature dependence of k_2 and k_4 was measured at pH 6 (for procedure see Lynch, 1973; Lynch and Schimmel, 1974). Values of 30 and 39 kcal mol⁻¹ were obtained for the activation energies for the $\text{X}_1 \rightarrow \text{X}_2$ and $\text{X}_3 \rightarrow \text{X}_4$ conversions, respectively. Essentially the same values for each rate constant and activation energy were obtained in both 10 mM Na⁺ and 45 mM Na⁺. At pH 7.5 the two activa-

tion energies are less—27 and 10^4 cal mol⁻¹, respectively (Lynch and Schimmel, 1974). The tendency for the activation energies to be somewhat higher at pH 6 is consistent with the notion that the presumed aberrant structure which is formed in low salt and the absence of Mg²⁺ (Cole *et al.*, 1972; Lynch and Schimmel, 1974) is somewhat more stable at pH 6 than at pH 7.5 due to the "abnormal" protonations. Since X₁ → X₂ and X₃ → X₄ conversions are believed to represent the breakdown of aberrant structures on the pathway to the native form (Lynch and Schimmel, 1974), it is reasonable that activation energies for these steps should be higher at pH 6 where the aberrant forms have greater stability.

Finally, the question was also raised as to the effect of other cations in facilitating the folding of tRNA. Both Na⁺ and spermidine³⁺ were tried as alternatives to Mg²⁺. It was found that addition of these ions gave rise to rate process and activation energies similar to those observed with Mg²⁺.

Environment of Probe. It is of interest to attempt to determine the environmental states of the probe which bring about the remarkable emission changes induced by varying pH and metal ion concentration. This is perhaps best accomplished by first comparing the emissions of the labeled tRNA and its fragments on the same absolute scale, and by fluorescence lifetime and polarization measurements on the derivatized tRNA under various conditions.

Figure 9 places the fluorescence pH titrations of intact tRNA (IV) and fragment II on the same scale. The relative positions of the curves were assigned on the basis of an experiment in which IV and II were obtained at the same concentration by directly converting IV in solution to II with RNase A. It is apparent from this figure that the conformational change induced in the tRNA by the addition of Mg²⁺ at pH 7.5 has the effect of elevating the probe's quantum yield to almost its value on II. This suggests that the conformational change induced by the addition of Mg²⁺ concludes with the 3'-terminus of the tRNA in a conformation similar to that of II, i.e., exposed to the solution and not interacting with the tRNA structure to any great extent. Therefore, the probe (and the 3'-terminus) in the low salt form is probably undergoing some interaction with the tRNA which results in fluorescence quenching.

Further support for the conclusion that in the high salt, high pH form of IV the probe is freely exposed to solution comes from fluorescence lifetime and polarization data. The fluorescence lifetimes of both I and IV were measured at pH 7.5 and 10 mM Mg²⁺. The apparent lifetimes for I and IV are 13 ± 1 and 10 ± 1 nsec, respectively. The similarity in lifetime for I and IV implies a similar quantum yield, which suggests a similar environment for each species.

The fluorescence polarizations of I, II, and the high salt, high pH form of IV are indistinguishable from background (i.e., zero polarization). Since all three have similar quantum yields (and lifetimes) this implies that the probe attached to the 3'-end of IV is rotating in solution independently of the whole tRNA molecule. In contrast, IV has a polarization of ca. 0.06 at pH 7.5 in 15 mM Na⁺, and a polarization of ca. 0.15 at pH 3.7 in 10 mM Na⁺. However, it is difficult to measure directly the lifetimes or polarized decays of the low salt forms, because at the concentrations required for sufficient emission, scattering causes serious interference. The lifetimes of the low salt species can be estimated from the lifetime of the high salt, high pH form (10 nsec) and the relative emission intensities of the different species (see Becker, 1969). From these data, it is estimated that the rotational unit associated with the probe in the low salt forms is significantly greater

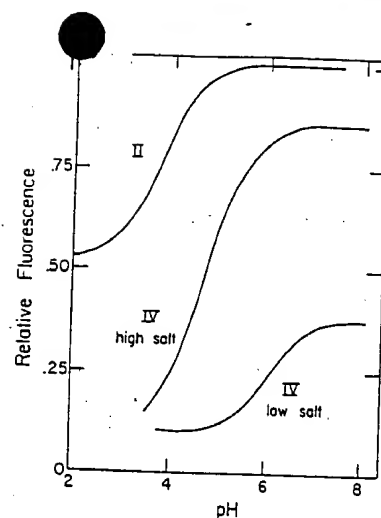


FIGURE 9: Sketch of titration curves of II and IV with and without Mg²⁺. There is no difference in the curves for II. The curves for IV are from Figure 3a.

than the volume of the fluorophore itself (Lynch, 1973; also, unpublished calculation). Hence, the probe is at least somewhat immobilized in the low salt species. Finally, it is not possible to reach the low pH fluorescence plateau at high [Mg²⁺] because of tRNA precipitation, and definitive polarization data on the low pH form were not obtained.

If the probe in its highly quenched state is somewhat immobilized on the tRNA, what is its environment? This question was investigated by studying the emission of I in various nonaqueous solvents. With the exception of 95% ethanol (which caused no quenching), all of the solvents used showed some quenching relative to an aqueous solution. Isopropyl alcohol, 1-butanol, dioxane, tetrahydrofuran, and ethyl ether all caused a moderate quenching of 15–30%. A chloroform-ethanol mixture (2:1 by volume) quenched two-thirds of the fluorescence, and a CCl₄-ethanol mixture (1:1 by volume) quenched 99% of the fluorescence. In all cases, the emission maximum remained substantially unchanged. Thus, it would appear that while a general quenching occurs in nonaqueous environments, the specific effects of the chlorocarbon solvents cause the more drastic quenching similar to that found in titrating tRNA from a high salt, high pH state to a low salt, low pH form. On the basis of these data, it is not possible to assign a probable location to the probe's interaction with the tRNA structure, other than to suggest that the quenching might result from a specific interaction (such as with a phosphate group) rather than a general effect caused by a nonaqueous environment.

Discussion

The data obtained by the fluorescence probe have indicated a remarkable effect, hitherto unrecognized, of pH upon the cooperative binding of Mg²⁺ to tRNA near neutral pH. Earlier studies which demonstrated cooperative Mg²⁺ binding were done above pH 7 where the pH effects are not evident (Cohn *et al.*, 1969; Danchin and Guéron, 1970; Danchin, 1972; Schreier and Schimmel, 1974). The single salt dependent pK_H detected by fluorescence ranges from pK_H = 6 (low salt) to pK_H = 4.6 (high salt). It is probably due to a cytidine residue near the probe and is doubtless typical of other "abnormal" ionizations on the tRNA. The effects of pH on the Mg²⁺ binding arise because protonation lends increased stability to the low salt, Mg²⁺-free form of tRNA. A prime result of these protonations is to make the Mg²⁺-induced uni-

molecular steps ($X_1 \rightarrow X_2$ and $X_3 \rightarrow X_4$, see eq 3) associated with breakdown of the low salt structure less facile at pH 6 as opposed to pH 7.5. The net effect is to reduce both the cooperativity and affinity of Mg^{2+} binding and to slow down the rate of folding of tRNA into its native structure, when Mg^{2+} is added to a low salt form.

It is of interest to draw a more concrete picture of the mode of action of the probe and its sensitivity to structural changes in the tRNA. A logical possibility is that it intercalates into the tRNA under certain conditions, since it is known that certain planar aromatic molecules have a tendency to intercalate into nucleic acid helices. The binding of proflavine to DNA has been extensively studied by Li and Crothers (1969) and the binding of ethidium to tRNA has been characterized by several workers (Bittman, 1969; Tao *et al.*, 1970; Tritton and Mohr, 1973). These molecules bind quite well to the nucleic acids (with dissociation constants of 10^{-5} – 10^{-6} M), but both are positively charged, a fact which accounts for much of their binding strength. For the binding of proflavine to DNA, Li and Crothers (1969) were able to resolve a two-step mechanism in which the first step represents binding of the proflavine to the outside of the DNA helix, and the second step represents the intercalation of proflavine into the helix. For the intercalation step they found an equilibrium constant of about 10 in favor of intercalation. It can be envisioned that in low salt the probe, in the present study, is involved in an intercalation which results in fluorescence quenching and polarization. This intercalation might occur in the amino acid acceptor helix. The way in which the probe is expelled could be quite subtle. Folding of the tRNA into a tertiary structure could, for example, cause a slight change in the pitch of the acceptor helix which might in turn cause decreased intercalation of the probe. Thus, the degree of emission of transient intermediates such as X_2 could be determined by the distribution of the probe between its free and bound states. In any event, one of the reasons for the great utility of the naphthoxyl probe in this work must lie in a gentle and easily altered mode of interaction with the tRNA.

Finally, the question of the generalization of these results to other tRNAs is of interest since all results were obtained with a specific species, tRNA^{11e}. To answer this question, some experiments were carried out with derivatized tRNA^{A1a} (*E. coli*). Preliminary experiments showed that the emission of the probe attached to this tRNA is also sensitive to Mg^{2+} and pH in a manner analogous to that found with tRNA^{11e} (Lynch, 1973). This suggests that the results reported in this and the preceding paper (Lynch and Schimmel, 1974), could be rather general.

Appendix I

Derivation of Rate Equations for Eq 3. The rate equations for the two slow steps of eq 3 may be derived by the procedure outlined in Lynch and Schimmel (1974). It is assumed that the free Mg^{2+} concentration is constant during the kinetic events (see Lynch and Schimmel, 1974). The two rate equations are

$$-d(\Delta HR + \Delta R + \Delta X_1)/dt = k_2 \Delta X_1 - k_{-2} \Delta X_2 \quad (I-1)$$

$$-d\Delta X_4/dt = -k_4 \Delta X_3 + k_{-4} \Delta X_4 \quad (I-2)$$

and the conservation equation among tRNA species is

$$\Delta HR + \Delta R + \Delta X_1 + \Delta X_2 + \Delta X_3 + \Delta X_4 = 0 \quad (I-3)$$

where ΔX_i is the deviation of the concentration of X_i from its final equilibrium value. The various equilibrium constant

relations for the steps which are rapid compared to the two slow ones, may be differentiated to give relationships among the various species. (For example, $K_H = [R][H^+]/[RH]$ and $\Delta RH = ([H^+]/K_H)\Delta R$, where it is assumed $\Delta H^+ = 0$ owing to buffering.) These relationships together with eq I-1 enable elimination of all terms except ΔX_1 and ΔX_4 from eq I-1 and I-2 with the result

$$-d\Delta X_1/dt = a_{11}\Delta X_1 + a_{12}\Delta X_4 \quad (I-4a)$$

$$-d\Delta X_4/dt = a_{21}\Delta X_1 + a_{22}\Delta X_4 \quad (I-4b)$$

where

$$a_{11} = \frac{k_2}{1 + K_1/[Mg^{2+}][1 + [H^+]/K_H]} + \frac{k_{-2}}{1 + [Mg^{2+}]/K_3} \quad (I-5a)$$

$$a_{12} = \frac{k_{-2}}{[1 + (K_1/[Mg^{2+}])][1 + [H^+]/K_H][1 + [Mg^{2+}]/K_3]} \quad (I-5b)$$

$$a_{21} = \frac{k_4[1 + (K_1/[Mg^{2+}])][1 + [H^+]/K_H]}{(1 + K_3/[Mg^{2+}])} \quad (I-5c)$$

$$a_{22} = \frac{k_4}{1 + K_3/[Mg^{2+}]} + k_{-4} \quad (I-5d)$$

The solution to eq I-4a,b involves finding the λ_i 's of eq (of the text) which are the eigenvalues of the determinant formed from the a_{ij} 's of eq I-4a,b. These are

$$\lambda_{1,2} = \frac{(a_{11} + a_{22}) \pm \sqrt{(a_{11} + a_{22})^2 + 4(a_{12}a_{21} - a_{11}a_{22})}}{2} \quad (I-6)$$

where λ_1 corresponds to the top sign and λ_2 to the bottom sign.

Rate constants may be extracted from the various limiting forms of the λ_i 's as discussed in Lynch and Schimmel (1974).

Appendix II

Derivation of θ in Terms of Fluorescent Species. Relative fluorescence values may be assigned to all of the species in eq 3 by the same procedure as used in Lynch and Schimmel (1974). The values at pH 6 (10 mM Na⁺) are

$$f_{HR} = 0.33f_R$$

$$f_{X_1} = f_R$$

$$f_{X_2} = 1.16f_R \quad (II-1)$$

$$f_{X_3} = 1.16f_R$$

$$f_{X_4} = 2f_R$$

The initial fluorescence F_i and final fluorescence F_f are given by

$$F_i = [HR]_i f_{HR} + [R]_i f_R \quad (II-2)$$

$$F_f = [R_0] f_{X_4} = 2[R_0] f_R \quad (II-3)$$

where $[R_0] = [RH] + [R] + [X_1] + [X_2] + [X_3] + [X_4]$. The fluorescence F at any concentration of Mg^{2+} is

$$F = [HR] f_{HR} + [R] f_R + [X_1] f_{X_1} + [X_2] f_{X_2} + [X_3] f_{X_3} + [X_4] f_{X_4} \quad (II-4)$$

Using eq II-1 and the definition of θ we obtain

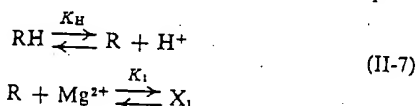
$$\theta = \frac{F - F_i}{F_f - F_i} = \frac{2[X_1] + 3[X_2] + 3[X_3] + 8[X_4]}{8[R_0]} \quad (II-5)$$

where the fact that $[R] = [RH]$ at pH 6 has been used. Dividing through by $[R]$ gives

$$\theta = \frac{\frac{2[Mg^{2+}]}{K_1} + \frac{3[Mg^{2+}]}{K_1 K_2} + \frac{3[Mg^{2+}]^2}{K_1 K_2 K_3} + \frac{8[Mg^{2+}]^2}{K_1 K_2 K_3 K_4}}{8 \left(2 + \frac{[Mg^{2+}]}{K_1} + \frac{[Mg^{2+}]}{K_1 K_2} + \frac{[Mg^{2+}]^2}{K_1 K_2 K_3} + \frac{[Mg^{2+}]^2}{K_1 K_2 K_3 K_4} \right)} \quad (II-6)$$

A log $((1 - \theta)/\theta)$ vs. pMg line may be generated with eq II-6, by using the values of K_1 , K_2 , K_3 , and K_4 obtained from the kinetic curves. The line so generated is linear over the range of $0.05 < \theta < 0.95$. The n and pK_{app} values obtained are 1.32 and 4.73, respectively, which agrees well with the observed values of 1.26 and 4.84, respectively. Although additional binding beyond the X_4 stage could be taken into account, the calculated behavior agrees satisfactorily enough without considering it.

The amplitude of the rapid jump in fluorescence, $\Delta F'$, shows a sigmoidal dependence on pMg. It is possible to derive an expression for the fractional values of this fluorescence jump, $\theta_j = \Delta F'/\Delta F'_{max}$. This may be done by considering the steps



Using the partial fluorescence of eq II-1 and the equality of $[R]$ and $[RH]$ at pH 6, we obtain

$$\begin{aligned} F_i &= f_{HR}[HR]_i + f_R[R]_i = 2f_{HR}[R]_i \\ F_t &= f_{X_1}[X_1]_t = 3f_{HR}[R]_i \\ F &= f_{HR}[HR] + f_R[R] + f_{X_1}[X_1] = 4f_{HR}[HR] + 3f_{HR}[X_1] \end{aligned} \quad (II-8)$$

where $[R]_i = [RH] + [R] + [X_1]$. The expression for θ_j is

$$\theta_j = \frac{F - F_i}{F_t - F_i} = \frac{[X_1]}{[RH] + [R] + [X_1]} \quad (II-9)$$

or

$$\theta_j = \frac{1}{(K_1/[Mg^{2+}]) [1 + ([H^+]/K_H)] + 1} \quad (II-10)$$

where $K_H = 10^{-6} = [H^+]$ at pH 6. It is clear from eq II-10 that it is possible to obtain K_1 directly from the observed midpoint of the $\Delta F'$ vs. $[Mg^{2+}]$ curve. See text for further discussion.

References

Alberty, R. A., Smith, R. M., and Bock, R. M. (1951), *J. Biol. Chem.* 193, 425.

- Azumi, T., and M. Lynn, S. P. (1962), *J. Chem. Phys.* 37, 2413.
- Becker, R. S. (1969), *Theory and Interpretation of Fluorescence and Phosphorescence*, New York, N. Y., Wiley.
- Beers, R. F., Jr., and Steiner, R. F. (1957), *Nature (London)* 179, 1076.
- Bittman, R. (1969), *J. Mol. Biol.* 46, 251.
- Cavalieri, L. F., and Stone, A. L. (1955), *J. Amer. Chem. Soc.* 77, 6499.
- Chen, R. F., and Bowman, R. L. (1965), *Science* 147, 729.
- Cohn, M., Danchin, A., and Grunberg-Manago, M. (1969), *J. Mol. Biol.* 39, 199.
- Cole, P. E., Yang, S. K., and Crothers, D. M. (1972), *Biochemistry* 11, 4358.
- Cox, R. A., and Peacocke, A. R. (1957), *J. Chem. Soc.*, 4724.
- Danchin, A. (1972), *Biopolymers* 11, 1317.
- Danchin, A., and Guéron, M. (1970), *Eur. J. Biochem.* 16, 532.
- Eigen, M., and Hammes, G. G. (1960), *J. Amer. Chem. Soc.* 82, 5951.
- Hartman, K. A., Jr., and Rich, A. (1965), *J. Amer. Chem. Soc.* 87, 2033.
- Hayes, N. V., and Branch, G. E. K. (1943), *J. Amer. Chem. Soc.* 65, 1555.
- Jordan, D. O. (1960), *The Chemistry of the Nucleic Acids*, Washington, D. C., Butterworths.
- Jordan, D. O., Mathieson, A. R., and Matty, S. (1956), *J. Chem. Soc.*, 158.
- Laitinen, H. A. (1960), *Chemical Analysis*, New York, N. Y., McGraw-Hill.
- Langridge, R., and Rich, A. (1963), *Nature (London)* 198, 725.
- Li, H. J., and Crothers, D. M. (1969), *J. Mol. Biol.* 39, 461.
- Lynch, D. C. (1973), Ph.D. Thesis, M.I.T.
- Lynch, D. C., and Schimmel, P. R. (1974), *Biochemistry* 13, 1841.
- Rich, A., Davies, D. R., Crick, F. H. C., and Watson, J. D. (1961), *J. Mol. Biol.* 3, 71.
- Saneyoshi, M., Harada, F., and Nishimura, S. (1969), *Biochim. Biophys. Acta* 190, 264.
- Schreier, A. A., and Schimmel, P. R. (1974), *J. Mol. Biol.* (in press).
- Steiner, R. F., and Beers, R. F., Jr. (1959), *Biochim. Biophys. Acta* 33, 470.
- Tao, T., Nelson, J. H., and Cantor, C. R. (1970), *Biochemistry* 9, 3514.
- Tritton, T. R., and Mohr, S. C. (1973), *Biochemistry* 12, 905.
- Yarus, M., and Barrell, B. G. (1971), *Biochem. Biophys. Res. Commun.* 43, 729.

RYO.5, both contain material remaining a, amino acid ratios, in acid hydrolysate: Lys., His., Asp., Ser., Glu., Pro., Gly., Ala., Cys., Val., Leu., Tyr., (80%), amino acid ratios in performic acid oxidized sample, Lys., His., Asp., Thr., Ser., Glu., Pro., Gly., Ala., Cys(SO₃H), Val., Leu., Tyr.,

(29) Corrected for destruction during acid hydrolysis.

ratios of diagnostic amino fragment A/fragment C (Cys(SO₃H)/Leu) = 3.0; fragment fragment D (Cys(SO₃H)/Lys) = 3.0; fragment B contains no diagnostic residues.

Acknowledgments. The authors wish to express their appreciation to Judy Montibeller for the PCMB titrations and to Guirguis Rizk for the amino acid analyses.

Structure of the Borohydride Reduction Product of Photolinked 4-Thiouracil and Cytosine. Fluorescent Probe of Transfer Ribonucleic Acid Tertiary Structure

Donald E. Bergstrom and Nelson J. Leonard*

Contribution from the School of Chemical Sciences,
University of Illinois, Urbana, Illinois 61801. Received February 24, 1972

Abstract: 5-(4-Pyrimidin-2-one)cytosine, Pyo(4-5)Cyt (2), a photoproduct which can be isolated from the irradiation (335 nm) of certain *E. coli* transfer RNAs and irradiation (254 nm) of polycytidylic acid, deoxycytidine, and cytidine, is reduced by sodium borohydride to a fluorescent compound, 5-(4-pyrimidin-2-one)-3,6-dihydrocytosine, Pyo(4-5)hCyt (3). Catalytic oxygenation (Pt, O₂) converted Pyo(4-5)hCyt back to Pyo(4-5)Cyt. Treatment of Pyo(4-5)hCyt with aqueous acid gave 5-(4-pyrimidin-2-one)-3,6-dihydrouracil, Pyo(4-5)hUra (8), which could also be obtained by the treatment of 5-(4-pyrimidin-2-one)uracil, Pyo(4-5)Ura (7), with sodium borohydride. 5-(4-Pyrimidin-2-one)-4-thiouracil, Pyo(4-5)Sur (9), a photoproduct from irradiation (335 nm) of 4-thiouracil in aqueous solution, is reduced by sodium borohydride to 5-(4-pyrimidin-2-one)-4-thio-3,6-dihydrouracil, Pyo(4-5)hSur (10). The fluorescent nature of Pyo(4-5)hCyt provides a useful monitor of the photoreaction of tRNAs containing proximate 4-thiouridine and cytidine moieties.

A specific intramolecular photoreaction has been shown to occur between 4-thiouridine and a cytidine in *E. coli* tRNA^{Val} on irradiation at 335 nm.¹ Only those *E. coli* tRNAs known to possess a 4-thiouridine moiety in nucleoside position 8 and a cytidine in position 13 from the 5'-terminal end yield a photoproduct under these conditions. Evidence for the covalent cross-linking between the two nucleosides after photolysis of the intact *E. coli* tRNA^{Val} at 335 nm was provided by enzymic fragmentation sequence studies.¹ Subsequently the photochemically cross-linked binucleotide unit was isolated by the complete enzymic digestion of the irradiated tRNA.^{1b} The structure of the corresponding binucleoside photoproduct has recently been determined as 1.^{2a} Compound 1, 5-(1-β-D-ribofuranosyl-4-pyrimidin-2-one)cytidine, was obtained in fair yield by irradiating 4-thiouridine and cytidine in aqueous solution at 4° at 335 nm. The corresponding bipyrimidine product, 5-(4-pyrimidin-2-one)cytosine, Pyo(4-5)Cyt (2),³ resulted on photolysis (335 nm) of 4-thiouracil in the presence of cytosine.²

The nature of the photoreaction and the structure of the photoproduct may lead to the acquisition of important structural and functional information about tRNA. It has been shown⁴ that the photolytically cross-linked *E. coli* tRNA^{Val} can be charged with valine in the presence of its corresponding aminoacyl synthetase, although the affinity for the synthetase is decreased. The Val-tRNA^{Val} functions normally in a reconstructed *in vitro* protein-synthesizing system. Qualitatively similar results have been obtained in experiments with *E. coli* tRNA^{Arg} and tRNA^{Phe}.⁵

The susceptibility of 4-thiouridine in tRNA to borohydride reduction⁶ apparently led to an attempted borohydride reduction of the photolytically cross-linked tRNA. When the photoproduct was treated with sodium borohydride it was converted to a new, highly fluorescent compound with emission maximum 440 nm and excitation maximum 386 nm.⁷ The degree and

attached by covalent linkage from the 4 position to the 5 position of cytosine. Based on the bipyrimidine system of nomenclature, which is less indicative of the biochemical connotation and interest, 5-(4-pyrimidin-2-one)cytosine is 4-amino-4',5'-bipyrimidine-2,2'-(1*H*,1'*H*)-dione. Other abbreviations follow the new photochemistry symbolism, e.g., Pdo(4-5)Cyd (in place of Cyd-Srd^{2a}) for 5-(1-β-D-ribofuranosyl-4-pyrimidin-2-one)cytidine (1); Pyo(4-5)Ura for 5-(4-pyrimidin-2-one)-uracil (7); Pyo(4-5)Sur for 5-(4-pyrimidin-2-one)-4-thiouracil (9). The corresponding dihydro products obtained by treatment of 2, 7, and 9 with sodium borohydride are designated, respectively, as Pyo(4-5)-hCyt (3) (rather than Cyt-Sur_{red}), Pyo(4-5)hUra (8), and Pyo(4-5)hSur (10).

(4) M. Yaniv, A. Chestier, F. Gros, and A. Favre, *J. Mol. Biol.*, **58**, 381 (1971).

(5) L. Chaffin, D. R. Omilianowski, and R. M. Bock, *Science*, **172**, 854 (1971).

(6) P. Cerutti, T. W. Holt, and N. Miller, *J. Mol. Biol.*, **34**, 505 (1968).

(7) A. Favre and M. Yaniv, *FEBS (Fed. Eur. Biochem. Soc.) Lett.*, **17**, 236 (1971).

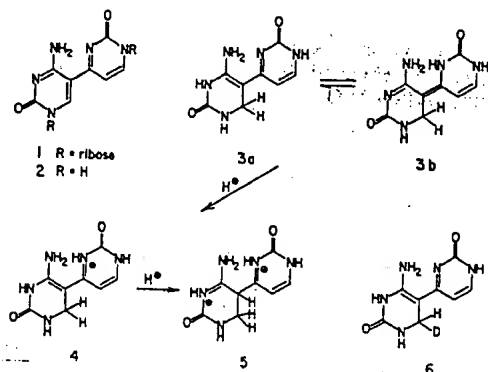
(1) (a) M. Yaniv, A. Favre, and B. G. Barrell, *Nature (London)*, **223**, 1331 (1969); (b) A. Favre, A. M. Michelson, and M. Yaniv, *J. Mol. Biol.*, **58**, 367 (1971).

(2) (a) N. J. Leonard, D. E. Bergstrom, and G. L. Tolman, *Biochem. Biophys. Res. Commun.*, **44**, 1524 (1971); (b) D. E. Bergstrom and N. J. Leonard, *Biochemistry*, **11**, 1 (1972).

(3) Abbreviations following the IUPAC-IUB Commission on Biochemical Nomenclature recommendations (*J. Mol. Biol.*, **55**, 299 (1971)) are used throughout. The photochemistry symbolism employed in ref. 1 has been modified and improved after discussions with Dr. Waldo Cohn, Director of the Office of Biochemical Nomenclature. For example, the earlier abbreviation, Cyt-Sur, for the photoproduct 2, which indicated the source of the two fragments, has been replaced by Pyo(4-5)Cyt, which represents the actual structure, now that it is known.² Pyo stands for pyrimidin-2-one and 4-5 indicates that it is

rate of photochemical cross-linking can be followed conveniently by treating the irradiated NA with sodium borohydride and measuring the relative fluorescent intensity at 440 nm.

The reduced photoproduct takes on added significance with the recent discovery that Pdo(4-5)Cyt (1) is a major photoproduct of the irradiation of polycyclic acid at pH 4.⁸ Although the importance of the formation of the photoproduct in RNA, or of the related, deoxyribose photoproduct in DNA, has yet to be determined, borohydride treatment of photolyzed RNA and DNA may allow detection of the cross-linked photoproduct at very low concentration levels.



We are now able to report the synthesis and structure of a fluorescent compound 3 identical with the product formed on borohydride reduction of cross-linked tRNA. The reduced photoproduct 3 was obtained by treatment of Pyo(4-5)Cyt (2) with sodium borohydride. In addition, the structures of the products formed on borohydride reduction of 5-(4-pyrimidin-2-one)uracil, Pyo(4-5)Ura (7), and 5-(4-pyrimidin-2-one)-4-thiouracil, Pyo(4-5)Sur (9), have been determined. The synthesis of Pyo(4-5)Ura by irradiation of a dilute aqueous solution of uracil and 4-thiouracil at 4° and of Pyo(4-5)Sur by irradiation (335 nm) of 4-thiouracil at 4° has been described.^{2b} Although neither of these bipyrimidine photoproducts has yet been found in irradiated tRNA, they have served as valuable analogs in the study of Pyo(4-5)Cyt (2).

Results

Sufficient quantities of Pyo(4-5)Cyt (2), Pyo(4-5)Ura (7), and Pyo(4-5)Sur (9) were necessary in order to characterize their borohydride reduction products. Pyo(4-5)Cyt can be prepared in low yield by direct photolysis of an aqueous solution of cytosine and 4-thiouracil; however, it was more efficiently prepared (70% yield) by treatment of Pyo(4-5)Sur with sodium metaperiodate in an aqueous ammonium ion buffer solution at pH 9.8. If the periodate reaction was attempted in the normal two steps, as for the conversion of 2'-deoxy-4-thiouridine to 2'-deoxycytidine via an intermediate sulfonate,⁹ it failed. Pyo(4-5)Sur (9) was easily obtained by photolysis of 4-thiouracil as previously described.^{2b} When 4-thiouracil was photolyzed in aqueous solution with a twofold excess of uracil the major product was Pyo(4-5)Ura (7).^{2b}

When Pyo(4-5)Cyt (2) was reduced with excess sodium borohydride in aqueous solution a single major product was isolated ($\lambda_{\text{max}}^{\text{H}_2\text{O}}$ 374 nm) (Figure 1). On

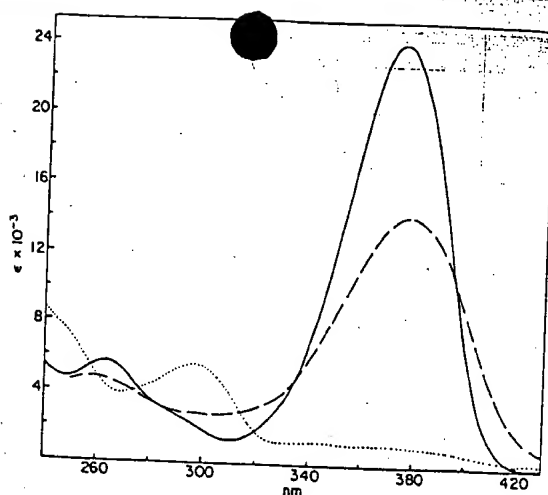
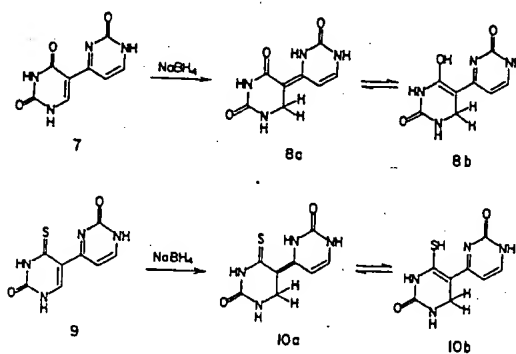


Figure 1. Ultraviolet spectrum of Pyo(4-5)hCyt (3) in 0.04 M HCl-10% DMSO-H₂O (---), 10% DMSO-H₂O (—), and 0.005 M NaOH-10% DMSO-H₂O (···); cf. Figure 1a in ref 7.



the basis of spectral data and elemental analysis the product was assigned structure 3. If one can extrapolate from the stable tautomeric forms of cytosine and 2-pyrimidinone, either one, or both, of the tautomeric forms 3a and 3b can be present. This point could not be clarified readily from the first spectral data. The reduced product, Pyo(4-5)hCyt (3), was found to have the composition C₈H₉N₃O₂ by elemental analysis, confirmed by the molecular ion M⁺ at *m/e* 207 observed in the low-resolution mass spectrum.

The nmr spectrum of 3 in trifluoroacetic acid was complex and unrewarding. However, the spectrum was somewhat simplified in trifluoroacetic acid-*d*₁, and peaks were observed which apparently arise from the presence of two different protonated forms of 3. The minor component showed a pair of doublets at δ 7.19 and 8.43 (*J* = 6.5 Hz) assignable to the C-5 and C-6 protons of the 2-pyrimidinone moiety, and a pair of doublets at δ 4.04 and 4.24 (*J* = 14.5 Hz) indicative of nonequivalent geminal protons. The major component showed a singlet at δ 4.37, a doublet at 7.45 (*J* = 7.5 Hz), and an unresolved multiplet at 6.76. For comparison, the nmr spectrum of the unreduced photoproduct, Pyo(4-5)Cyt, showed a singlet at δ 8.99 due to the C-6 proton of the cytosine moiety and two doublets at 7.37 and 8.38 (*J* = 7.5 Hz) assignable to the C-5 and C-6 protons of the 2-pyrimidinone moiety. The disappearance of the C-6 proton resonance at δ 8.99, retention of low-field doublets¹⁰ between 6.5 and 8.5, and

(10) Although the peak at δ 6.76 appeared as a broad singlet in the 220-MHz spectrum, it sometimes appeared as a broad doublet (*J* = 7.5 Hz), especially in 60-MHz spectra. Such peak broadening has often been observed for the C-5 proton of cytosine derivatives. We have observed that the C-5 and C-6 protons of 4-methyl-2-pyrimidinone are

(8) D. F. Rhoades and S. Y. Wang, *Biochemistry*, 10, 4603 (1971).

(9) E. B. Ziff and J. R. Fresco, *J. Amer. Chem. Soc.*, 90, 7338 (1968).

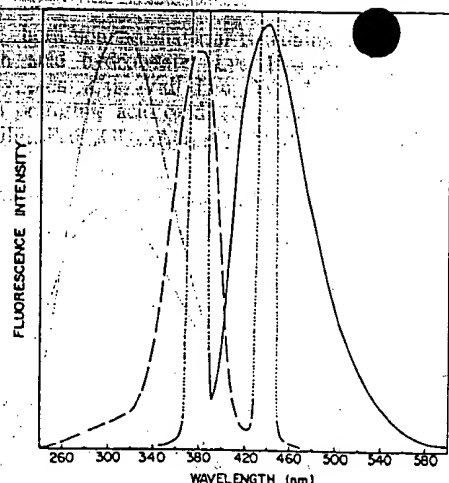


Figure 2. Technical fluorescence excitation (---) (λ_{em} 440 nm) and emission spectra (—) (λ_{ex} 386 nm) of Pyo(4-5)hCyt (3) in 1,2-propanediol at 20°. The peaks resulting from light scattering by the solvent are shown (···); cf. Figure 2 in ref 7.

the appearance of new signals at high field, integrating for two protons, suggested that hydride had added at C-6. The nmr spectrum of Pyo(4-5)hCyt in fluorosulfonic acid clarified the nature of the two components observed in the trifluoroacetic acid- d_1 spectrum. In fluorosulfonic acid two sharp doublets at δ 7.78 and 8.91 ($J = 6.5$ Hz) were assignable to the C-5 and C-6 protons, a broad singlet at 9.41 was assumed to be due to the two N-4 protons, and a broad singlet at 8.47 was assignable to the N-1 proton. An apparent doublet of doublets at δ 5.14 (J uncertain) had been observed previously in the nmr spectrum of Pyo(4-5)hCyt in trifluoroacetic acid as an unresolved multiplet at 4.91 which integrated for a single proton of the minor component. This peak was not observed in the nmr spectrum of Pyo(4-5)hCyt in trifluoroacetic acid- d_1 . The final peak in the fluorosulfonic acid spectrum was a complex multiplet at δ 4.40, integrating for two protons, which is explicable as part of an ABX pattern assignable to the two C-6 protons.¹¹ We conclude that Pyo(4-5)hCyt has the doubly protonated structure 5 in fluorosulfonic acid and that the singly protonated structure 4 is the major component of an equilibrium mixture of 4 and 5 in trifluoroacetic acid. Consistent with the singly protonated Pyo(4-5)hCyt structure 4, which would exist in a time-averaged planar conformation with the positive charge distributed over both rings (one contributor to the resonance hybrid is shown), is the singlet in the nmr spectrum for nondifferentiated protons at C-6. By contrast, the nonequivalence of the C-6 protons in the doubly protonated form requires a structure (5, one contributor to the resonance hybrid shown) in which the two rings are noncoplanar.

The nmr spectroscopic assignments were checked by the reduction of Pyo(4-5)Cyt (2) with sodium borodeuteride, which yielded a product 6 that gave a molecular ion M^+ at m/e 208 in the low-resolution mass spectrum. Whereas the borohydride reduction product 3 showed, *inter alia*, two doublets in the nmr spectrum taken in trifluoroacetic acid- d_1 (δ 4.04 and 4.24, $J = 14.5$

sharp doublets in $(CH_3)_2SO-d_6$, but in CF_3COOH are broadened to the extent that they appear as broad singlets similar to the δ 6.76 resonance for compound 3.

(11) Resolution of the multiplet was too poor to obtain accurate coupling constants.

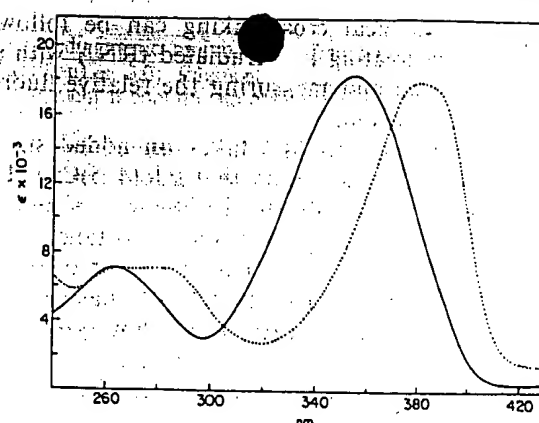


Figure 3. Ultraviolet spectrum of Pyo(4-5)hUra (8) in 0.04 M HCl-10% DMSO-H₂O and 10% DMSO-H₂O (—), and 0.005 M NaOH-10% DMSO-H₂O (···).

Hz), corresponding to the doubly protonated species with deuterium at C-5, the borodeuteride reduction product 6 showed two singlets under the same conditions.

Compounds Pyo(4-5)Cyt and Pyo(4-5)hCyt were found to be interconvertible. Thus, compound 3 could be oxidized back to 2 by platinum and oxygen in aqueous solution. Precedent for the oxidation was found in the catalytic oxygenation of the borohydride-reduced *cis-syn*-thymine photodimer back to the *cis-syn*-thymine dimer.¹²

Many of the difficulties encountered in studying the spectroscopic properties of Pyo(4-5)hCyt arose because of its low solubility in solvents in which it was stable. A neutral solvent for the observation of the nmr spectrum would have been especially desirable, but, for instance, the low solubility (0.5 mg/ml) of Pyo(4-5)hCyt in dimethyl sulfoxide was only sufficient to allow preparation of solutions for quantitative uv spectra. Although compound 3 was readily soluble in aqueous base or acid, decomposition was evident in both media. The product resulting from treatment of 3 with 1 *N* hydrochloric acid was characterized as 5-(4-pyrimidin-2-one)-3,6-dihydrouracil, Pyo(4-5)hUra (8), and was identical with the product obtained on borohydride reduction of Pyo(4-5)Ura (7).

The fluorescence emission of Pyo(4-5)hCyt at 440 nm was examined in different solvents and was found to increase in the order: aqueous solution at neutral and basic pH, 4% dimethyl sulfoxide-ethanol, 1,2-propanediol. In aqueous acid, Pyo(4-5)hCyt was not fluorescent. Even in 1,2-propanediol the intensity of the fluorescence was slight in comparison with the intensity of the solvent scattering at the wavelength of excitation (374 nm) (Figure 2). The absolute quantum efficiencies of Pyo(4-5)hCyt in 1,2-propanediol and water were determined to be 0.013 and approximately 0.001, respectively, by integration and comparison of the peak areas of the corrected emission spectra with the area of the emission peak for quinine sulfate¹³ obtained with the same instrument settings.

Treatment of Pyo(4-5)Ura (7) with sodium borohydride in aqueous solution gave a white solid with λ_{max}^{UV} 355 nm (Figure 3) and composition $C_8H_8N_4O_3$ by ele-

(12) T. Kunitada and B. Witkop, *J. Amer. Chem. Soc.*, **93**, 3493 (1971).

(13) T. G. Scott, R. D. Spencer, N. J. Leonard, and G. Weber, *ibid.*, **92**, 687 (1970).

mental analysis. A low-resolution mass spectrum showed a molecular ion M^+ at 208, which was also the base peak in the 9-eV mass spectrum. These data, along with an unambiguous nmr spectrum (CF_3COOH), which showed a singlet at δ 4.36 for the C-6 methylene protons, doublets at 5.96 and 7.38 ($J = 7.5$ Hz) assignable to the C-5 and C-6 protons at the 2-pyrimidinone moiety, and two broad singlets at 7.40 and 9.32 assignable to the N-1 and N-3 protons of the uracil portion of the molecule,¹⁴ led us to assign structure 8 to the borohydride reduction product of Pyo(4-5)Ura.

Reduction of Pyo(4-5)Sur (9) with sodium borohydride gave 5-(4-pyrimidin-2-one)-4-thio-3,6-dihydrouracil, Pyo(4-5)hSur (10). The assigned structure followed from spectral data and elemental analysis. Neither Pyo(4-5)hUra nor Pyo(4-5)hSur was fluorescent in aqueous solution. Although we have not distinguished between the two most likely tautomeric forms for Pyo(4-5)hUra (8a and 8b) and Pyo(4-5)hSur (10a and 10b) by spectroscopic means, tautomers 8a and 10a are favored for two reasons. First, on the basis of past experience, the keto and thioketo forms of the oxygen- and sulfur-substituted pyrimidines are favored over the enol and thioenol forms. Second, the large hypsochromic shift (61 nm) in the uv maximum of Pyo(4-5)hSur (λ_{max} 427.5 nm, Figure 4) when it is oxidized to a disulfide by aqueous iodine suggests that thioketone conjugation was responsible for the long-wavelength absorption of 10. In analogy the uv maxima of 4-thiouridine and its disulfide are 328 and 309 nm, respectively.¹⁵ 1,4-Dithiothreitol (Cleland's reagent) regenerates 10 from its disulfide.

Discussion

5-(4-Pyrimidin-2-one)cytosine, Pyo(4-5)Cyt (2), has been identified as the major photoproduct (at the base level) from photolysis of *E. coli* tRNA at 335 nm,^{1,2} and irradiation of polycytidylic acid, deoxycytidine, and cytidine at pH 4 and 254 nm.⁸ The formation of 1, and of 2 by subsequent hydrolysis, by a photoreaction between the 4-thiouridine in position 8 and the cytidine in position 13 from the 5'-terminal end of *E. coli* tRNA_{Val}, tRNA_{2A}^{Val}, tRNA_{2B}^{Val}, tRNA^{Phe}, tRNA_{Met}, tRNA_{Met},¹ and tRNA^{Arg}⁵ is of interest because of the detailed information it provides concerning the tertiary structure in the dihydrouridine-arm region of the molecule.^{2b} Moreover, an added dividend is the facility with which Pyo(4-5)Cyt can be converted to a fluorescent derivative, Pyo(4-5)hCyt (3), by treatment with sodium borohydride. The structure proof of 5-(4-pyrimidin-2-one)-3,6-dihydrocytosine (3) implies a similar structure, ribose-substituted at both 1 positions, for the sodium borohydride reduction product of 5-(1- β -D-ribofuranosyl-4-pyrimidin-2-one)cytidine, Pdo(4-5)Cyd (1), and thus settles the question of the structure of the reduced cross-linked moiety in tRNA. We recognize, of course, that the reactivity of Pyo(4-5)Cyt may be altered by change in environment from base to nucleoside to polynucleotide levels.

The introduction of a covalently bonded fluorescent probe in tRNA is clearly of interest for studying tRNA

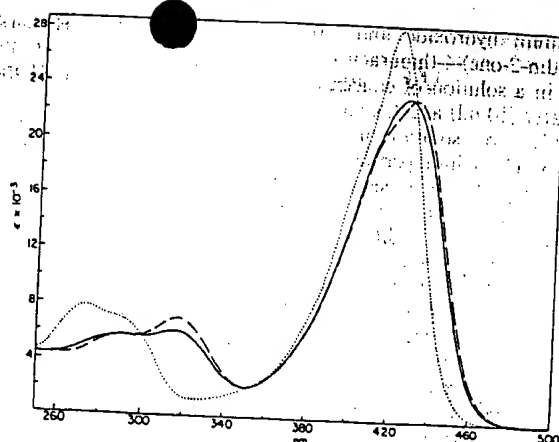


Figure 4. Ultraviolet spectrum of Pyo(4-5)hSur (10) in 0.04 M HCl-10% DMSO-H₂O (---), 10% DMSO-H₂O (—), and 0.005 M NaOH-10% DMSO-H₂O (···).

conformation and interaction.⁷ In addition, the borohydride reduction of Pdo(4-5)Cyd (1) or Pyo(4-5)Cyt (2) provides a sensitive detection method for the photoproduct. The excitation and emission spectra of reduced photoproduct in tRNA_{Val} have been reported⁷ and the quantum yield and fluorescent lifetime measured ($22 \pm 5\%$ and <5 nsec). When the photoproduct was first isolated from tRNA_{Val} as a binucleotide and then reduced, the fluorescence quantum yield in aqueous solution was estimated to have decreased by a factor of 400.^{1b,7} The absolute quantum efficiencies of 0.013 and approximately 0.001 for Pyo(4-5)hCyt which we observed in 1,2-propanediol and water, respectively, indicating that the fluorescence intensity of Pyo(4-5)hCyt increases with decreasing solvent polarity, are consistent with Favre and Yaniv's conclusion⁷ that Pyo(4-5)hCyt must lie within a hydrophobic region in the transfer RNA. The fluorescence of Pyo(4-5)hCyt spotted on cellulose tlc plates and viewed under long-wavelength uv light is intense enough to allow ready visual detection of as little as 2-5 ng (10 pmol).

Sodium borotritide reduction has been used for the quantitative determination of dihydrouridine, 4-thiouridine, and *N*⁴-acetylcytidine in tRNA,⁶ and of cyclobutane photodimers in polynucleotides and deoxyribonucleic acid.¹⁶ For the detection of small amounts of Pyo(4-5)Cyt quantitatively, reduction with sodium borotritide would give Pyo(4-5)hCyt tritiated at C-6. One may speculate that the amount of photoproduct could then be determined from the ³H activity of any of a number of isolated products: Pyo(4-5)hUra by direct degradation of the irradiated nucleic acid with 1 N HCl, Pdo(4-5)hCyd by an enzymic isolation procedure, or Pyo(4-5)Cyt by the catalytic oxygenation (O_2 , Pt) of Pyo(4-5)hCyt in the nucleic acid followed by acid degradation. Since it is of further interest to determine the importance of Pyo(4-5)Cyt to the photobiology of DNA,⁸ the fluorescent product of borohydride reduction should also aid in these studies.

Experimental Section¹⁷

5-(4-Pyrimidin-2-one)cytosine, Pyo(4-5)Cyt (2). A buffer solution, pH 9.8, was prepared by combining equal volumes of 7.4 M

(14) For comparison, the N-1 and N-3 protons of dihydrouracil (CF_3COOH) fall at δ 7.34 and 9.36, respectively.

(15) J. J. Fox, D. Van Praag, I. Wempen, I. L. Doerr, L. Cheong, J. E. Knoll, M. L. Eidinoff, A. Bendich, and G. B. Brown, *J. Amer. Chem. Soc.*, **81**, 178 (1959).

(16) (a) T. Kunieda and B. Witkop, *ibid.*, **89**, 4232 (1967); (b) T. Kunieda, L. Grossman, and B. Witkop, *Biochem. Biophys. Res. Commun.*, **33**, 453 (1968); (c) B. Witkop, *Photochem. Photobiol.*, **7**, 813 (1968).

(17) Melting points, determined using a Büchi melting point apparatus, are uncorrected. Ultraviolet spectra were taken in dimethyl sulfoxide-water (1:9, v/v) with a Cary 15 spectrophotometer and technical

ammonium hydroxide and 8.0 M ammonium chloride. 5-(4-Pyrimidin-2-one)-4-thiouracil (9) (58.5 mg, 0.1 mmol) was dissolved in a solution of concentrated ammonium hydroxide (1 ml) and water (10 ml) and the solution was diluted with pH 9.8 buffer (100 ml). To a solution of pH 9.8 buffer (100 ml), water (25 ml), and 0.3 M sodium periodate (10 ml), the buffered solution of Pyo(4-5)Sur (9) was added dropwise over a period of 1 hr. After stirring an additional hour at room temperature the reaction mixture was stored overnight at 5° and filtered, and the solid product was collected and washed thoroughly with water. The crude product was dissolved in 0.1 M HCl (18 ml), filtered, and reprecipitated by neutralization of the solution with ammonium hydroxide. Filtration and drying *in vacuo* gave 41.1 mg of Pyo(4-5)Cyt·H₂O (70%). The identity of Pyo(4-5)Cyt (2) prepared in this way with that obtained photochemically from cytosine and 4-thiouracil¹ was shown by thin-layer chromatography in three different solvent systems, ultraviolet spectra in acidic, basic, and neutral aqueous solution, and direct comparison of physical properties and solubility behavior.

5-(4-Pyrimidin-2-one)-3,6-dihydrocytosine, Pyo(4-5)hCyt (3). 5-(4-Pyrimidin-2-one)cytosine (2) (16.8 mg, 0.075 mmol) was dissolved in 0.14 N HCl (7 ml) and diluted to 150 ml with water (distilled under nitrogen). To the vigorously stirred solution under nitrogen was added 1 M NaOH (1.0 ml) followed immediately by aqueous 1 M NaBH₄ (1.0 ml). The reaction mixture was stirred for 45 min at room temperature and cooled for 30 min in an ice bath, and 1 ml of acetone was added to decompose the unreacted borohydride. The cold reaction mixture was filtered, and the pale yellow precipitate was washed thoroughly with cold water to give, after drying, 11.8 mg of 3 (76%): mp >340° dec; nmr (CF₃COOD) showed two components to be present, minor with δ 4.04 (d, 1, J = 14.5 Hz), 4.24 (d, 1, J = 14.5 Hz), 7.19 (d, 1, J = 6.5 Hz), 8.43 (d, 1, J = 6.5 Hz); major with δ 4.37 (s, 2), 6.76 (br m, 1), 7.45 (d, 1, J = 7.5 Hz); nmr (FSO₃H) δ 4.40 (m, 2), 5.14 (d of d, 1, J uncertain), 7.78 (d, 1, J = 6.5 Hz), 8.47 (br s, 1), 8.91 (d, 1, J = 6.5 Hz), 9.41 (br s, 2); tlc, R_f in system A, 0.20; B, 0.21; C, 0.29; λ_{\max} 374 nm (ϵ 24,300), 264 (5760); $\lambda_{\max}^{0.005 M HCl}$ 377 (14,300), 259 (4820); $\lambda_{\max}^{0.005 M NaOH}$ 368 (1260), 344 (1340), 297 (5690). The mass spectrum (70 eV) showed prominent peaks at m/e (rel intensity) 207 (5), 150 (7), 149 (36), 148 (22), 135 (6), 124 (8), 123 (9), 122 (11), 121 (15), 120 (8), 105 (7), 96 (12), 95 (8), 94 (9), 80 (5), 79 (5), 69 (5), 68 (14), 67 (6), 66 (5), 55 (5), 54 (6), 53 (8), 52 (13), 51 (6), 44 (22), 43 (100), 42 (33), 41 (8), 40 (8), 39 (6), 32 (5), 29 (17), 28 (44), 27 (10), 26 (6); (8.7 eV) m/e (rel intensity) 207 (47), 178 (22), 176 (17), 167 (15), 166 (79), 152 (70), 150 (26), 149 (100).

Anal. Calcd for C₈H₉N₃O₂: C, 46.38; H, 4.38; N, 33.80. Found: C, 46.12; H, 4.43; N, 33.45.

5-(4-Pyrimidin-2-one)-3,6-dihydrocytosine-6-*d*₁ (6). 5-(4-Pyrimidin-2-one)cytosine (2) (34.5 mg, 0.168 mmol) was reduced with sodium borodeuteride (70.2 mg, 1.68 mmol) essentially as described above for the sodium borohydride reduction, to give 6 (23.9 mg, 68% yield): mp >340° dec; λ_{\max} 374 nm; nmr (CF₃COOD) showed two components to be present, minor with δ 4.04 (s, 0.5), 4.24 (s, 0.5), 7.20 (d, 1, J = 6.5 Hz), 8.44 (d, 1, J = 6.5 Hz); major with δ 4.36 (s, 1), 6.84 (d, 1, J = 7.5 Hz), 7.46 (d, 1, J = 7.5 Hz); R_f values in three solvent systems were identical with the R_f values of Pyo(4-5)hCyt (3). The mass spectrum (70 eV) showed prominent peaks at m/e (rel intensity) 208 (3), 152 (5), 151 (20), 150 (41), 149 (32), 148 (14), 125 (8), 124 (10), 123 (14), 122 (15), 121 (12),

fluorescence emission spectra with a Hitachi Perkin-Elmer MPF-2A fluorescence spectrophotometer. Absolute quantum efficiencies were determined by integration of the corrected spectra obtained with a digital spectrofluorometer as previously described.¹² As a reference the absolute quantum efficiency of quinine sulfate was taken as 0.70.¹² Proton magnetic resonance (pmr) spectra were determined on a Varian HA-100 or HR-220 spectrometer with tetramethylsilane (TMS) or tetramethylammonium fluoroborate as the internal standard. The tetramethylammonium fluoroborate resonance in fluorosulfonic acid was taken as δ 3.10.¹³ The low-resolution mass spectral data were obtained on a MAT CH-5 spectrometer. Thin-layer chromatography (tlc) was carried out on 200 × 40 × 0.16 mm Eastman Chromagram sheets, cellulose without fluorescent indicator, in the following solvent systems: A, *n*-propyl alcohol-water (7:3, v/v); B, ethanol-1.0 M ammonium acetate (7:3, v/v), buffered to pH 7.95 with concentrated NH₄OH; C, *n*-propyl alcohol-concentrated NH₄OH-water-formic acid (60:29:10:1), v/v. Spots were visualized by long-wavelength uv light. Elemental microanalyses were performed by Mr. Josef Nemeth and his associates at the University of Illinois and by Midwest Microlab, Inc., Indianapolis, Ind.

(18) (a) N. C. Deno, H. G. Richey, Jr., N. Friedman, J. D. Hodge, J. J. Houser, and C. U. Pittman, Jr., *J. Amer. Chem. Soc.*, **85**, 2991 (1963); (b) N. C. Deno, J. S. Liu, J. O. Turner, D. N. Lincoln, and R. E. Fruit, Jr., *ibid.*, **87**, 3000 (1965).

107 (5), 106 (6), 97 (6), 95 (13), 94 (5), 81 (5), 80 (6), 79 (6), 69 (6), 68 (16), 67 (7), 55 (7), 53 (10), 52 (12), 44 (9), 43 (100), 42 (20), 41 (8), 40 (10), 29 (14), 28 (23), 27 (5); mass spectrum (8.7 eV) m/e (rel intensity) 208 (14), 168 (13), 167 (22), 152 (22), 151 (42), 150 (100), 149 (29).

5-(4-Pyrimidin-2-one)-3,6-dihydrouracil, Pyo(4-5)hUra (8). To a solution of 5-(4-pyrimidin-2-one)uracil (7) (90.5 mg, 0.44 mmol) in 300 ml of 0.007 M NaOH was added sodium borohydride (100 mg, 2.64 mmol). The reaction mixture was buffered to pH 9 with 0.5 M KH₂PO₄ and stirred for 30 min at room temperature, and a further portion of sodium borohydride (20 mg, 0.53 mmol) was added. After stirring 15 min more at room temperature the reaction mixture was buffered to pH 7 with 0.5 M KH₂PO₄, and the excess NaBH₄ was decomposed by adding 1 ml of acetone. The light yellow precipitate which formed on storing the solution overnight at 5° was collected and dried *in vacuo* to give 66.4 mg (73%) of 8. The compound was obtained analytically pure by recrystallization from CF₃COOH-CH₃COOH: mp >340°; nmr (CF₃COOH) δ 4.36 (s, 2), 5.96 (d, 1, J = 7.5 Hz), 7.38 (d, 1, J = 7.5 Hz), 7.40 (br s, 1), 9.32 (s, 1); R_f in system A, 0.43; B, 0.41; C, 0.48; λ_{\max} 355 nm (ϵ 17,600), 263 (7100); $\lambda_{\max}^{0.005 M HCl}$ 355 (18,300), 263 (7100); $\lambda_{\max}^{0.005 M NaOH}$ 380 (16,700), 280 (5830), 266 (5830). The mass spectrum (70 eV) showed prominent peaks at m/e (rel intensity) 208 (42), 207 (100), 164 (48), 137 (25), 136 (34), 135 (17), 122 (12), 121 (33), 113 (22), 108 (16), 96 (30), 95 (12), 94 (12), 93 (12), 82 (14), 68 (27), 67 (23), 66 (23), 65 (11), 54 (10), 53 (16), 52 (26), 51 (11), 44 (25), 43 (31), 42 (13), 41 (15), 40 (19), 39 (15), 32 (13), 29 (10), 28 (74), 27 (11); mass spectrum (9 eV) m/e (rel intensity) 209 (13), 208 (100), 207 (38), 206 (11), 113 (4), 96 (5).

Anal. Calcd for C₈H₈N₂O₂: C, 46.16; H, 3.87; N, 26.91. Found: C, 46.25; H, 3.94; N, 27.10.

5-(4-Pyrimidin-2-one)-4-thio-3,6-dihydrouracil, Pyo(4-5)hSur (10). To a solution of 5-(4-pyrimidin-2-one)-4-thiouracil (9) (41.3 mg, 0.186 mmol) in 20 ml of 0.1 M ammonium bicarbonate (pH 9.1) was added sodium borohydride (32.2 mg, 0.85 mmol). The reaction mixture was stirred for 10 min at room temperature, then quenched by adding consecutively 1 ml of acetone and 5 ml of 0.5 M H₂PO₄, and placed in an ice bath for 2 hr. The precipitate was collected and dried to give 29.5 mg (71%) of 10. Analytically pure 10 was obtained by chromatography on Sephadex LH-20 and elution with *N,N*-dimethylformamide: mp >340° dec; nmr (CF₃COOH) δ 4.39 (s, 2), 6.13 (d, 1, J = 7.5 Hz), 7.30 (br s, 1), 7.47 (d, 1, J = 7.5 Hz), 9.32 (s, 1); R_f in system A, 0.59; B, 0.50; C, 0.59; λ_{\max} 427.5 nm (ϵ 23,500), 316 (6110), 289.5 (5770); $\lambda_{\max}^{0.005 M HCl}$ 431 (23,300), 316 (6950), 291 (5650); $\lambda_{\max}^{0.005 M NaOH}$ 423 (28,400), 273 (7860); mass spectrum (70 eV) m/e (rel intensity) 224 (17), 223 (6), 191 (5), 73 (11), 68 (9), 62 (6), 60 (100), 59 (5), 44 (21), 43 (87), 42 (19), 34 (18), 33 (8), 32 (45), 30 (6), 29 (15), 28 (65), 27 (8), 26 (5).

Anal. Calcd for C₈H₈N₂O₂S: C, 42.85; H, 3.60; N, 24.98. Found: C, 43.13; H, 3.64; N, 24.73.

Oxidation of Pyo(4-5)hCyt (3) to Pyo(4-5)Cyt (2). Active platinum was prepared by reducing platinum oxide (18.6 mg) with H₂ in 25 ml of water. A suspension of the active platinum and compound 3 (3.4 mg, 0.016 mmol) was prepared in 25 ml of water. After sparging with oxygen for 1 min at room temperature, 2.0 ml of 1 N HCl was added to the reaction mixture. The oxygen sparging was discontinued after 15 min, the reaction mixture was filtered, and the water was removed *in vacuo*. The white residue was collected, washed thoroughly with ethanol, and dried to give 3.5 mg (89%) of 2 as the hydrochloride salt. The quantitative uv spectra of the product at acidic, neutral and basic pH were identical with those of authentic Pyo(4-5)Cyt.² The identity of the compound was further established by comparative tlc in three solvent systems and by its rereduction to Pyo(4-5)hCyt with sodium borohydride.

Hydrolysis of Pyo(4-5)hCyt (3) to Pyo(4-5)hUra (8). A solution of Pyo(4-5)hCyt (1.1 mg) in 1 N HCl (0.8 ml) was allowed to stand at room temperature overnight. The white precipitate which formed was collected, washed with water, and dried *in vacuo* yielding 1.0 mg of Pyo(4-5)hUra. The identity of Pyo(4-5)hUra prepared in this way with that obtained from the sodium borohydride reduction of Pyo(4-5)Ura was shown by tlc in three different solvent systems, uv spectra in acidic, basic, and neutral aqueous solution, and by the mass spectrum.

Acknowledgment. We wish to thank Professor Gregorio Weber for valuable guidance and for the use of the digital spectrofluorometer. This work was supported by a research grant (GP-8407X) from the National Science Foundation.

of different RNA species was detected. As presently constituted, the system is designed to fractionate a mixture of components of equal chain length according to their base composition. This is because the per cent cross-linking in the gels is low and consequently they have a large pore size. It should be possible to change the pore size so that both molecular size and net charge are used to effect separation.

ACKNOWLEDGMENT

The patient and skillful technical assistance of B. V. Coakley is gratefully acknowledged.

REFERENCES

- 1 G. M. Tener, in S. P. Colowick and N. O. Kaplan, *Methods in Enzymology*, Vol. 12A, Academic Press, New York, 1967, p. 398.
- 2 G. W. Rushizky, E. M. Bartos and H. E. Sober, *Biochemistry*, 3 (1964) 626.
- 3 F. Sanger, G. G. Brownlee and B. G. Bartell, *J. Mol. Biol.*, 13 (1965) 373.
- 4 C. G. Brownlee and F. Sanger, *Eur. J. Biochem.*, 11 (1969) 395.
- 5 E. M. Southern and A. R. Mitchell, *Biochem. J.*, 123 (1971) 613.
- 6 G. W. Rushizky and H. A. Sober, *Biochem. Biophys. Acta*, 55 (1962) 217.
- 7 M. Uziel, in S. P. Colowick and N. O. Kaplan, *Methods in Enzymology*, Vol. 12A, Academic Press, New York, 1967, p. 407.
- 8 H. C. Birnboim and J. Glickman, *J. Chromatog.*, 44 (1969) 581.
- 9 H. C. Birnboim and B. V. Coakley, *Biochem. Biophys. Res. Commun.*, 42 (1971) 1169.
- 10 B. G. Lane, *Biochemistry*, 4 (1965) 212.
- 11 K. Burton, in S. P. Colowick and N. O. Kaplan, *Methods in Enzymology*, Vol. 12A, Academic Press, New York, 1967, p. 222.
- 12 U. E. Loening, *Biochem. J.*, 102 (1967) 251.
- 13 E. G. Richards and R. Lecanidou, *Anal. Biochem.*, 40 (1971) 43.
- 14 H. C. Birnboim, *Anal. Biochem.*, 29 (1969) 498.
- 15 T. Clausen, *Anal. Biochem.*, 22 (1968) 70.
- 16 K. Daneck, G. Weimann and F. Cramer, *Experientia*, 21 (1965) 417.
- 17 J. J. Butzow and G. L. Eichhorn, *Biochemistry*, 10 (1971) 2019.

Biochim. Biophys. Acta, 269 (1972) 217-224

QUANTITATIVE TECHNIQUE FOR MAPPING OLIGONUCLEOTIDES ON THIN LAYERS OF CELLULOSE

H. W. MUNDY AND H. PRIESS

Max-Planck-Institut für Biologie, Abt. Melchers, Tübingen (Germany)
Received December 1st, 1971

SUMMARY

A procedure for two-dimensional separation of oligonucleotides on thin layers of cellulose is described. It implies electrophoretic separation for the first dimension and chromatography for the second. Oligonucleotides of α -³²P-labelled material are localized by autoradiography. The procedure is particularly useful for mapping oligonucleotides from digests of RNA with pancreatic ribonuclease, but is also applicable to non-radioactive nucleotides. It produces well reproducible and characteristic fingerprints in which the structure of an oligonucleotide can be deduced from its position on the map in many cases. Oligonucleotides can be eluted about 5 can be separated, depending on the complexity of the sample. They can be eluted quantitatively and counted. Their nucleotide composition is determined by electrophoretic separation of the mononucleotides resulting from hydrolysis with alkali or better, mixtures of ribonucleases T_1 and T_2 , and quantization of the mononucleotides after elution. Since the results are also quantitatively reproducible, the method is suited for compiling quantitative oligonucleotide catalogs of larger RNA segments.

INTRODUCTION

Earlier successes in the sequence analysis of nucleotides in RNA solely dependent on the fractionation of oligonucleotides of various digests of RNA on chromatographic columns^{1,2}. In 1965 two-dimensional fractionation procedures were published which initiated remarkable progress in this field, in particular the use of cellulose acetate and DEAE paper by Sanger and his collaborators³, and of DEAE-cellulose thin layers by the same group⁴.

Older methods based on chromatography and/or electrophoresis on thin layers of non-substituted celluloses^{5,6}, remained inferior to comparable paper techniques in resolution and quantitative recovery of oligonucleotides. The more recent and much advanced two-dimensional techniques involve a transfer^{7,8,9} of material from one carrier (cellulose acetate) to another (DEAE- or PEI-cellulose), which is not al-

Abbreviation: TMV, tobacco mosaic virus RNA

K. W. MUNDY, H. PRIEST
ways. In spite of the splendid results obtained with these methods, compilation of quantitative oligonucleotide catalogs sometimes requires other procedures.

We want to report a technique for mapping oligonucleotide mixtures on commercial cellulose thin-layer chromatographic plates, which is greatly improved compared to methods already existing for this material and which lacks a transfer step thus permitting quantitative investigations. In a number of cases the method has in our hands proven to be easier to handle than the other methods mentioned and to be quantitatively very well reproducible. It has already been successfully applied to RNA¹⁰.

MATERIALS AND METHODS

Thin-layer equipment for fingerprints

Precoated thin-layer chromatographic plates (0.1 mm layer of microcrystalline cellulose on 20 cm x 20 cm glass plates, without fluorescence indicator, type 5716) were obtained from Merck, Darmstadt, Germany. An electrophoresis apparatus in design very similar to the one commercially available through Desaga, Heidelberg, Germany, was used. It is a flat-plate apparatus with a water-cooled aluminum block supporting the glass plates. With this apparatus it is necessary to protect the thin layers against drying during electrophoresis by a 20 cm x 20 cm gasket of 1-cm-wide strips of foam rubber of sufficient thickness to provide a sealed moist chamber between the layer and the lucite cover of the apparatus. Chromatography was performed in thin-layer chromatographic Chromatanks of Shandon, Ltd, London.

Thin-layer equipment for electrophoretic separation of mononucleotides

This was the already mentioned Desaga type of apparatus in case pre-coated glass plates were used (see above). Alternatively, we used pre-coated thin-layer chromatographic plastic sheets (0.1 mm layer of fibrous cellulose on 20 cm x 20 cm plastic sheets, with fluorescence indicator, type Polygram CEL 300 UV₂₅₄) purchased from Macherey and Nagel, Düren, Germany. Electrophoresis on plastic sheets was carried out in a custom-made apparatus of proper dimensions and basically similar in construction to the one described for sheets of filter paper by Rushizky and Knight¹¹ but filled with varsol and equipped with a copper coil (connected running tap water) to cool the varsol.

Chemicals

These were of reagent grade whenever available. Pancreatic ribonuclease (lyophilized, phosphate-free, RAF 6507) was obtained from Worthington, Freehold, N.J., and ribonuclease T₁ from the Sankyo Co., Tokyo. Ribonuclease U₂ was a generous gift of the latter company. ³²P-labelled tobacco mosaic virus (TMV) RNA was prepared as described previously¹⁰. The specific activity was usually between 0.15 and 0.35 μ Ci/ μ g of RNA (corresponding roughly to $4.5 \cdot 10^6$ – $10 \cdot 10^6$ Cerenkov counts per A_{260} nm unit).

FINGERPRINTING OF OLIGONUCLEOTIDES

Autoradiographs

Autoradiographs were obtained with Kodak Royal Blue RB 54 X-ray film. A time of 5 h/50 000 dpm per spot can be taken as a guide for proper exposure with a ³²P-containing material.

Hydrolysis with ribonucleases

RNA samples (0.1–1.0 μ g equalling 50 000 dpm or more of ³²P, solved in 0.2 M lithium acetate with 0.5% sodium dodecyl sulphate, and contained in 1.5 ml polythene centrifuge tubes) were precipitated with alcohol, washed several times with 70% alcohol and dried for 10 min (not more) at room temperature *in vacuo*. Hydrolysis with pancreatic ribonuclease was performed by dissolving the dried sample in 7–10 μ l of 0.01 M Tris-acetate and 1 mM EDTA, pH 7.4, containing 0.2 μ g of ribonuclease per μ l and incubation for 60 min at 37°C. These conditions have proven to be practically optimal for the RNA quantities mentioned.

Hydrolysis with ribonuclease T₁ was performed in 0.04 M Tris-phosphate, pH 7.4, containing 1 mM EDTA, at an enzyme to substrate ratio of about 1:20 for 40 min at 37°C in a volume of 5–10 μ l. T₁ ribonuclease was usually treated with acid as a precaution against possible contamination with phosphomonoesterase¹².

Hydrolysis with ribonuclease U₂ was performed in 0.02 M sodium acetate with 1 mM EDTA, pH 3.8, similar to Adam *et al.*¹³. Since the enzyme is purine-specific only at low enzyme concentrations^{12,13}, we used low concentrations so as to reach sufficient hydrolysis within 4–8 h incubation at 37°C. We did not establish standard conditions for hydrolysis with ribonuclease U₂, though good results were obtained with 5–10 μ l of an enzyme solution containing 10 units/ml buffer, and 2 or 4 h hydrolysis at 37°C for an amount of substrate between 0.1 and 1.0 μ g of RNA.

Fingerprint technique

The plates were prepared by removing the cellulose layer about 1.5 cm wide from two opposite edges (see Figs 1b, 1d and 1f). The plate is then sprayed with a 1:1 aqueous dilution of electrophoresis buffer (see below) and placed horizontally. Several minutes later excess liquid is removed by gently pressing three sheets of Kleenex tissue, on top of which one sheet of filter paper, onto the cellulose. The sample (5–10 μ l) is spotted on the wet plate as a band 5–10 mm long, apart from two edges and usually parallel to the non-scraped edges of the plate (see Figs 1b, 1d and 1f, and, as an exception, Fig. 2b). Marker spots containing dye markers according to Sanger *et al.*³ were applied at the center and near the two scraped edges of the plate in line with the starting band. Electrophoresis buffer is 20% acetic acid with 8% formamide, adjusted with concentrated ammonia to pH 3.5. Electrophoresis is started immediately and carried out parallel to the scraped edges at 750 V. The current rises from about 20 mA at the beginning to about 35 mA during the run. Higher currents usually indicate that the plates are too wet. Electrophoresis is discontinued when the front of the blue dye marker (Xylene Cyanol F.F.)³ has moved 8.0–8.5 cm in case of pancreatic ribonuclease digests, 7.0–7.5 cm in case of T₁ ribonuclease digests, and 6.5–7.0 cm with ribonuclease U₂ hydrolysates. After removing the plates from the apparatus they are dried under a stream of slightly warm air for at least 3 h. Chromatography is performed in thin-layer chromatographic Chromatanks at 18–20°C, filled with 250 ml of a 1:1 mixture of

electrophoresis buffer with tertiary butanol, adjusted with concentrated ammonia to give a pH-meter reading of 4.5 (ref. 7). Higher temperatures and decreased butanol content of the developing mixture increase chromatographic movement but generally reduce the sharpness of the spots, also increase their size and thus reduce the general quality of the fingerprints.

When the solvent has reached the top of the plate, the plate is removed from the tank, dried as before, and re-chromatographed under the same conditions in the same direction. Chromatographic development may be repeated once more, again after proper drying. Finally the plate is dried and autoradiographed after asymmetric application of radioactive marker ink (see Figs. 1 and 2). This is prepared from a 1% solution of Xylene Cyanol blue to which sufficient α p has been added (see Table I).

TABLE I
RADIOACTIVITY OF AND EXPOSURE TIME FOR MARKER INK
Marker spots were about 3–5 mm in diameter.

dpm/ μ l	Time of exposure					
	3 h	6 h	12 h	1 day	3 days	6 days
6000	3000	1500	750	300	150	100

Spot elution and quantitative analysis

Spots are marked with soft pencil on the cellulose layer after superimposing the autoradiogram with the thin-layer chromatographic plate in front of an illuminated screen. They are scraped off the plates using a custom-made perspex spatula. The cellulose material is collected by sucking it into plastic pipette tips (see below) which are stoppered with cotton and connected to a vacuum pump. The tips are placed into small 1.5 ml polythene centrifuge tubes (see below) and the material is eluted and hydrolyzed according to one of the following two methods.

Method A. The tips are filled with 500 μ l of 10% piperidine in 0.1 mM EDTA and allowed to drain slowly, thus washing the oligonucleotide material into the polythene tubes. This elution is repeated once and then finally, excess liquid is extruded and collected by centrifugation into fresh polythene tubes. The polythene tubes are then placed in scintillation vials and the Cerenkov activity of the material is determined¹⁴ in the preset tritium channel of a Packard Tricarb Scintillation spectrometer Model 3375. Complete hydrolysis of the eluted material to mononucleotides takes place within 48 h at 56°C. The samples are then dried for several hours at 56°C under a fan. The dried samples still containing appreciable amounts of brown colored basic residue, are dissolved in 5 or 10 μ l of an aqueous solution containing 50 $A_{260 \text{ nm}}$ units/ml of each of the unlabelled nucleotides; Ap, Gp, Cp, and Up.

Method B. Elution is performed as described as *Method A* but with 0.2 M ammonia instead of the piperidine/EDTA mixture. After measuring the radioactivity (as before in *Method A*) the samples are degassed, frozen at -70°C and lyophilized, or are simply dried in an evacuated desiccator at room temperature without previous degassing and freezing. The dry residues (practically invisible small amounts) are

FINGERPRINTING OF OLIGONUCLEOTIDES

dissolved in 5 or 10 μ l of a mixture of ribonuclease T_1 and T_2 . A 5-fold dilution of the T_1/T_2 extract¹⁵ with 0.05 M sodium acetate, pH 4.5, is used, containing sufficient unlabelled RNA (5 mg/ml) to give visible mononucleotide bands on fluorescence layers (see below). Hydrolysis is allowed to proceed for 60 min at 37°C.

Electrophoresis of mononucleotides

Electrophoresis is carried out in the presence of dye markers³ on cellulose layers containing fluorescence indicators, either as done for the first dimension in the mapping procedure on glass plates, or, alternatively and better, on precoated plastic sheets submerged in varsol during electrophoresis. In the latter case a field of 100 V/cm and a current from 80 to 250 mA can be applied and excellent separation is achieved within 30 min. Not only is this separation procedure faster, but it also produces sharper bands and is more convenient than the one using glass plates (for comparison see Fig. 3). Its disadvantage, however, is that quantitative collection of α p from plastic sheets may be cumbersome because of electrostatic effects.

Mononucleotide bands are marked with soft pencil under ultraviolet light at 254 nm. The material is collected, eluted with ammonia or piperidine, and counted as described before, or counted by suspending the dry cellulose material directly in 5 ml of toluene-based scintillation cocktail.

Identification of nucleoside 2':3'-cyclic phosphates

This was done via a second hydrolysis of the eluted compound with the same enzyme, e.g. with pancreatic ribonuclease for Up and Cp, or with ribonuclease T_1/T_2 for Ap and Gp, followed by an electrophoretic comparison of the resulting material with an untreated aliquot.

General remark

During all these procedures components of the Eppendorf Microliter System have been used, in particular plastic pipette tips, polythene centrifuge tubes (1.5 ml), and piston-type "Marburg" pipettes. Some details of the elution technique are similar to a technique developed independently by Gassen⁶.

RESULTS AND DISCUSSION

The methods described in this paper yield two-dimensional separations of oligonucleotides, which are qualitatively and quantitatively reproducible. This is demonstrated with maps obtained from TMV-RNA hydrolyzed with three different ribonucleases.

The procedure is particularly useful for pancreatic ribonuclease hydrolysis products (Figs. 1a and 1b). The conditions of hydrolysis chosen avoid the occurrence of twin spots and clearly result in complete termination of the enzymatic reaction with the exception of the two mononucleotides Up and Cp, which still occur in varying amounts as their 2':3'-cyclic phosphodiester in addition to the phosphomonoester end product. On the other hand, "overhydrolysis"^{16,17} resulting in the release of Ap, ApAp, and also some ApApAp, cannot be avoided completely, but remains reasonably low.¹⁰

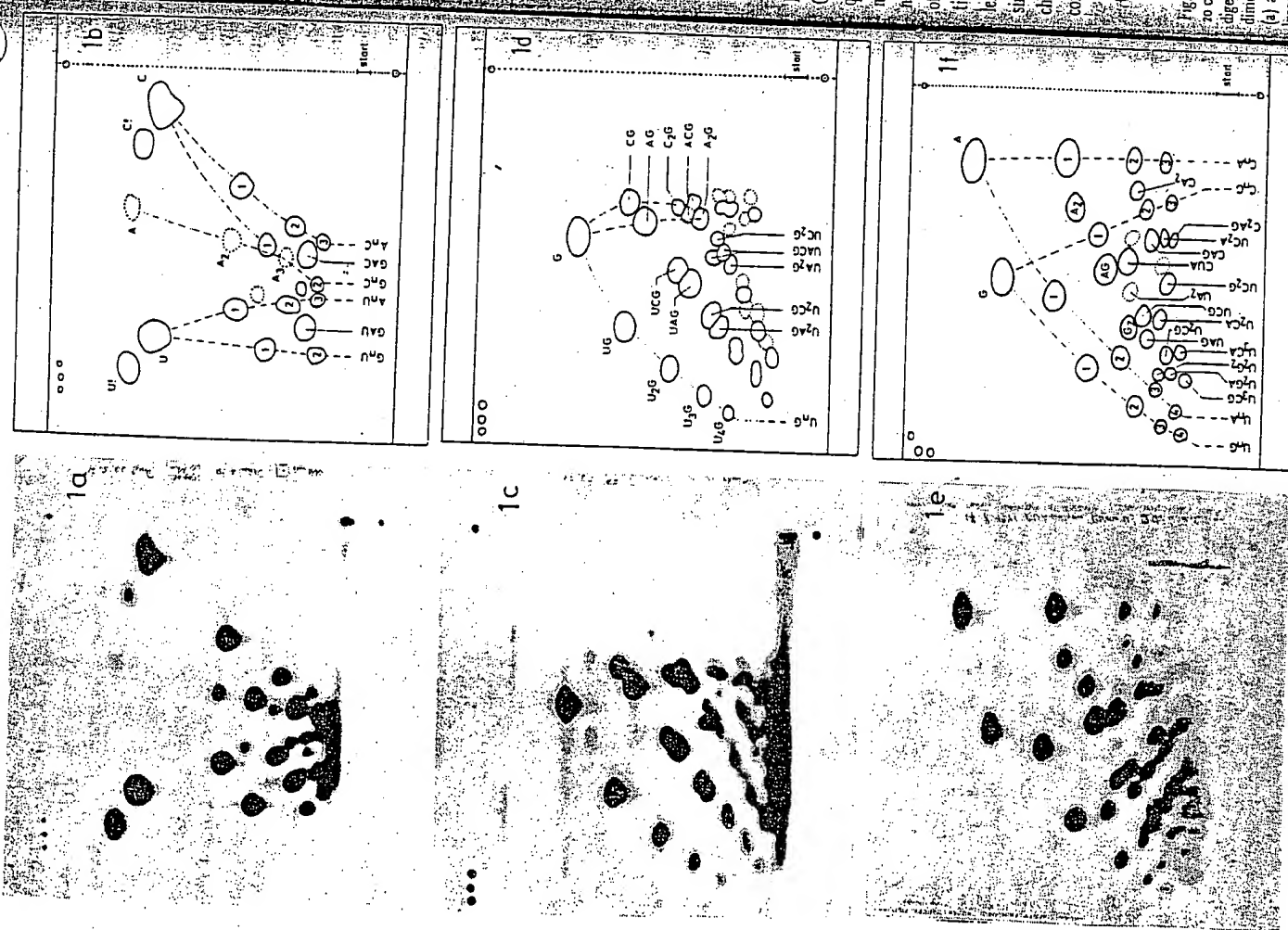


Fig. 1. Autoradiograms and corresponding oligonucleotide identification schemes for 20 cm x 20 cm thin-layer chromatographic cellulose plates with fingerprints from ³²P-labelled TMV-RNA digested with various ribonucleases. First dimension: electrophoresis from right to left, second dimension: ascending chromatography. For details of technique see Materials and Methods. (a) and (b) TMV-RNA hydrolyzed with pancreatic ribonuclease. (c) and (d) TMV-RNA hydrolyzed with ribonuclease T₁. (e) and (f) TMV-RNA hydrolyzed with ribonuclease U₂. Sequence of nucleotide symbols does not always correspond to actual nucleotide sequence in the oligomer. Many spots do contain isomers.

Oligonucleotide mixtures derived from RNA with ribonuclease T₁ (Figs. 1c and 1d) contain many chains longer than five nucleotides, which can be separated one from another only in special cases. Cyclic monophosphodiester are absent from most digests as are products of "overhydrolysis".

Oligonucleotide mixtures derived from RNA by digestion with ribonuclease U₂ (Figs. 1e and 1f) do not contain so many larger products and should, therefore, be rather suited for this type of mapping. However, mapping is complicated in this case by the small charge differences between A and C, which may lead to incomplete electrophoretic separation of A and C containing oligonucleotides, and also sometimes by spot duplication due to the presence of cyclic and open monophospho end groups.

Elution of oligonucleotides is practically quantitative with all spots obtained by this procedure and checked so far. Thus the activity remaining in the plastic chips after elution corresponded to only 0.5% of the eluted activity in the case of di-, and trinucleotides, to 1.5% in case of A₃C, and to about 5% for A₄C and the rather integral oligonucleotide mole ratios obtained in analyses from pancreatic ribonuclease digests of a very long oligonucleotide with the chain length of 73 (Table II) also demonstrate reasonable quantitative elution of the fingerprint spots. The regularity of low values for Cp cannot be explained so far. They are not due to insufficient elution of the Cp spots. The reason for the often somewhat too high values for ApUp (Table II) is at least partly due to some unidentified radioactive material comigrating with ApUp.

Hydrolysis of eluted oligonucleotides to mononucleotides and separation of the latter by thin-layer electrophoresis permits qualitative and quantitative determinations of nucleotide compositions. This is of interest where longer oligonucleotides of families as A_nPy, Py_mA_nG, or U_nPu, are to be identified, in particular within incomplete homologous rows (see below).

The procedure is, therefore, applicable to sequence analysis of polynucleotides. It has already enabled us to compile quantitative oligonucleotide catalogs of large G-lacking segments of TMV-RNA, where the main advantage of the method, the quantitative analysis, was of particular importance because of the large amounts of mononucleotides deriving from these segments by the action of pancreatic ribonuclease¹⁰. With an average from quantitative analyses of several maps an accuracy of $\pm 5\%$ per nucleotide spot is possible. This means that quantitative oligonucleotide catalogs can be compiled for molecules up to about 100 nucleotide residues in length. For example, in case of ribonuclease T₁ hydrolysis products of this size¹⁰ the single (terminal) Gp residue would amount to only 1% of the activity of the total chain. Thus chain length determinations based only on the Gp content would become less accurate with increasing chain length. However, exact determination of

TABLE II

CALCULATED OLIGONUCLEOTIDE MOLES (M) FOR VARIOUS ASSUMED CHAIN LENGTHS OF A LONG G-LACKING SEGMENT OF TMV-RNA, STRAIN VULGARE, HYDROLYZED WITH PANCREATIC RIBONUCLEASE

Radioactivity of individual fingerprint spots is calculated as a percentage of total activity of all spots. This value is then divided by the oligonucleotide size n , and reduced in proportion to the assumed chain length of the total segment. Δ is the difference between these oligonucleotide values and integral mole values. $\Sigma\Delta_{in}$ is the corresponding sum expressed in moles of phosphate. For plot of data see Fig. 4. Data are based on measurements given in ref. 10. The data in chain length of 73 best, since G must be present at 1 mole/mole RNA segment.

Oligonucleotide		3I		56		4I		46	
Structure	Size (n)	M	Δ	M	Δ	M	Δ	M	Δ
U	1	5.12	0.12	5.94	0.06	6.77	0.23	7.59	0.41
AU	2	1.80	0.20	2.09	0.09	2.38	0.38	2.67	0.31
AAU	3	1.67	0.33	1.94	0.06	2.21	0.21	2.48	0.48
C	1	0.66	0.34	0.76	0.24	0.87	0.13	0.98	0.02
AC	2	2.55	0.45	2.96	0.04	3.37	0.37	3.78	0.22
AAC	3	2.55	0.45	2.96	0.04	3.37	0.37	3.78	0.22
AAAC	4	0.86	0.14	0.99	0.01	1.13	0.13	1.27	0.27
G	1	0.45	0.55	0.52	0.48	0.60	0.40	0.67	0.33
$\Sigma\Delta_{in}$		5.21	1.38			4.52		5.04	

Oligonucleotide		5I		56		6I		66		7I	
Structure	Size (n)	M	Δ	M	Δ	M	Δ	M	Δ	M	Δ
U	1	8.42	0.42	9.24	0.24	10.07	0.07	10.89	0.11	11.72	0.28
AU	2	2.97	0.03	3.26	0.26	3.55	0.45	3.84	0.16	4.13	0.13
AAU	3	2.75	0.25	3.02	0.02	3.29	0.29	3.56	0.44	3.83	0.17
C	1	1.08	0.08	1.19	0.19	1.30	0.30	1.40	0.40	1.51	0.49
AC	2	4.19	0.19	4.61	0.39	5.02	0.02	5.43	0.43	5.84	0.16
AAC	3	4.19	0.19	4.60	0.40	5.01	0.01	5.42	0.42	5.83	0.17
AAAC	4	1.41	0.41	1.55	0.45	1.68	0.32	1.82	0.18	1.96	0.01
G	1	0.74	0.26	0.81	0.19	0.89	0.11	0.96	0.04	1.03	0.03
$\Sigma\Delta_{in}$		4.16	4.98			3.60		5.03		2.56	

chain lengths is possible within this size range when the quantitative determinations of all oligonucleotides of a map are given equal importance. This is the case when the sum of the deviations from possible integer values of the observed oligonucleotide moles is calculated for different chain lengths, expressed in mononucleotide moles and plotted. Applying this method chain lengths of large G-lacking segments isolated from TMV-RNA strains were found to be 56, 73, and 79 (83) nucleotides long (Table II and Fig. 4)¹⁰.

The basic principle of mapping oligonucleotides (that is separation according to base composition, namely by charge at pH 3.5 in the first dimension and by oligonucleotide chain length through chromatography in the second dimension) is very clearly demonstrated with a fingerprint obtained from a segment of TMV-RNA of the composition ($A_{38}U_{17}C_{13}$)G hydrolyzed with pancreatic ribonuclease. Homologous rows for oligonucleotides of the general structure A_nU and A_nC , and one member of the

Oligonucleotide		72		73		74		75	
Structure	Size (n)	M	Δ	M	Δ	M	Δ	M	Δ
U	1	11.88	0.12	12.05	0.05	12.22	0.22	12.38	0.38
AU	2	4.19	0.19	4.25	0.25	4.30	0.30	4.36	0.36
AAU	3	3.88	0.12	3.94	0.06	3.99	0.01	4.04	0.0
C	1	1.53	0.47	1.55	0.45	1.57	0.43	1.59	0.41
AC	2	5.92	0.08	6.01	0.01	6.09	0.09	6.17	0.17
AAC	3	5.91	0.09	5.99	0.01	6.08	0.08	6.16	0.16
AAAC	4	1.99	0.01	2.01	0.01	2.04	0.04	2.07	0.07
G	1	1.05	0.05	1.06	0.06	1.07	0.07	1.09	0.09
$\Sigma\Delta_{in}$		1.85		1.33		1.93		2.82	

Oligonucleotide		76		8I		86		9I		96	
Structure	Size (n)	M	Δ	M	Δ	M	Δ	M	Δ	M	Δ
U	1	12.55	0.45	13.37	0.37	14.20	0.20	15.02	0.02	15.85	0.15
AU	2	4.42	0.42	4.71	0.29	5.00	0.00	5.29	0.29	5.58	0.12
AAU	3	4.10	0.10	4.37	0.37	4.64	0.36	4.91	0.09	5.18	0.18
C	1	1.61	0.39	1.72	0.28	1.83	0.17	1.93	0.07	2.04	0.04
AC	2	6.25	0.25	6.66	0.34	7.07	0.07	7.49	0.49	7.90	0.10
AAC	3	6.24	0.24	6.65	0.35	7.06	0.06	7.47	0.47	7.88	0.12
AAAC	4	2.10	0.10	2.24	0.24	2.37	0.37	2.51	0.49	2.65	0.35
G	1	1.10	0.10	1.18	0.18	1.25	0.25	1.32	0.32	1.39	0.07
$\Sigma\Delta_{in}$		3.70		5.21		3.50		5.61		3.9	

family A_nG are to be expected, and a proper systematic distribution of these oligonucleotides has been found (Figs 2a and 2b). It is also evident from this and other fingerprints (Figs 1b and 1f) that Gp-containing oligonucleotides move somewhat more slowly during chromatography than G-lacking compounds of the same chain length. To some degree this applies similarly to Ap-containing and Ap-free oligonucleotides. The only other exception from the basic principle mentioned is that the 2':3'-cyclic monophosphodiester tend to migrate in both dimensions faster than their open monomer isomers.

The method described here has several advantages. One is the lack of a transfer of material from one carrier to another so that distortions of oligonucleotide ratios are avoided. Another advantage is its simplicity. The chromatographic step is easy to perform and requires no particular or costly equipment. The need for one or two repetitions of the chromatographic development may be regarded as a disadvantage

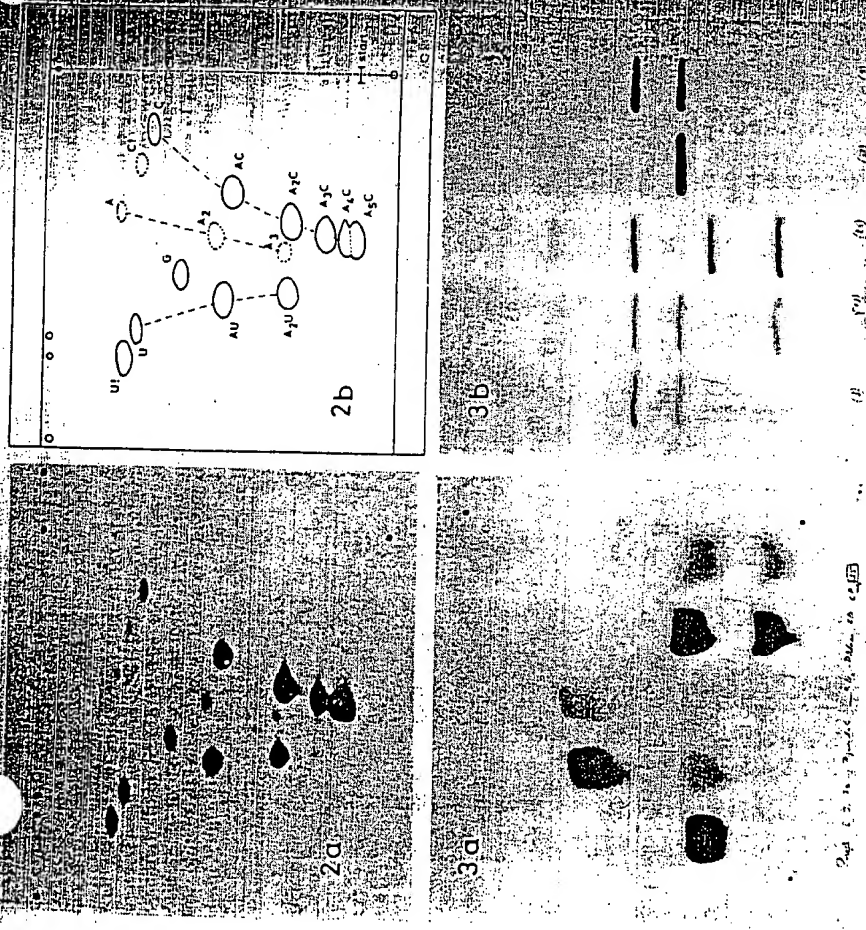


Fig. 2. Autoradiogram of oligonucleotide identification scheme for a fingerprint obtained with pancreatic ribonuclease from a G-lacking segment of TMV-RNA strain U2 with the general composition (A₃U₂C₃)G showing the homologous rows A₃U, A₃C, and A₃ (cited from ref. 10). The hydrolyzed oligonucleotides are (from left to right): U, C, G, UCA, G, and UG.

Fig. 3. Autoradiograms of separations of mononucleotides by electrophoresis on thin layers of cellulose at pH 3.5 in 20% acetic acid with 8% formamide. (a) According to Method A with alkaline digestion in 10% piperidine and electrophoresis on thin-layer chromatographic glass plates for 200 min at 40 V/cm. The hydrolyzed oligonucleotides are (from left to right): A, U, A, U, A, C, A, and Ap. (b) According to Method B with substrate hydrolyzed enzymatically with ribonuclease T₁ and T₂ and electrophoresis on thin-layer chromatographic plastic sheets submerged in varsol and run for 30 min at 100 V/cm. The hydrolyzed oligonucleotides are (from left to right): U₂C, UCG, UCA, G, and UG.

which so far cannot be circumvented by changes in the butanol content of the developing medium. A disadvantage is the limitation of the method for oligonucleotides of rather short chain length.

According to preliminary results (K. W. Mundry, unpublished) application of this method is not limited to radioactive material. The capacity of the cellulose thin layers appears sufficient to take 4-5 A₂₆₀ nm units of nucleotide material. With

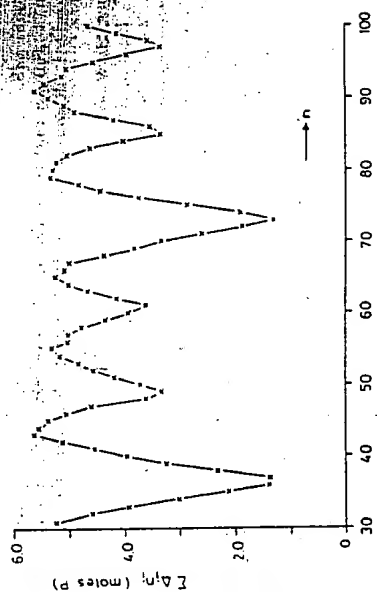


Fig. 4. Chain length determination for a long oligonucleotide. The sum of the deviations from possible integer values of oligonucleotide moles is expressed in moles phosphate and is plotted versus assumed total chain length (for details see Table II).

fluorescence layers, 0.1 A₂₆₀ nm unit produces a well visible spot. Provided the nucleic acid material is clean and free from other ultraviolet-absorbing material and assuming the structure to be analyzed would yield 25 spots, each spot would contain on average 0.2 A₂₆₀ nm unit of nucleic acid material and would, therefore, be easily detectable under ultraviolet light. With advanced techniques of nucleotide quantization^{18,19} quantitative analyses of eluted material appears possible.

ACKNOWLEDGEMENTS

We wish to thank Ulrike Oster and Wolfgang Klemisch for skilful technical assistance during this investigation and Ingrid Slama for help with spot identifications of some of the fingerprints. Programming the laboratory computer by Dr Vladimir Zarybnicky is cordially acknowledged. The generous gift of a lyophilized sample ribonuclease U₂ by courtesy of Mr Okazaki of the Sankyo Co., Tokyo, is very much appreciated, as is support by Prof. W. Zillig, Max-Planck-Institut für Biochemie, Munich, and by the Deutsche Forschungsgemeinschaft, Bad Godesberg.

REFERENCES

- 1 R. W. Holley, J. Appgar, G. A. Everett, J. T. Madison, M. Marquisee, S. H. Merrill, J. R. Penswick and A. Zamir, *Science*, **147** (1965) 1462.
- 2 H. G. Zachau, D. Düttling and H. Feldmann, *Hoppe Seyler's Z. Physiol. Chem.*, **347** (1966) 212.
- 3 F. Sanger, G. C. Brownlee and B. G. Barrell, *J. Mol. Biol.*, **13** (1965) 373.
- 4 G. G. Brownlee and F. Sanger, *Eur. J. Biochem.*, **11** (1969) 395.
- 5 K. Randerath and E. Randerath, in L. Grossman and K. Moldave, *Methods in Enzymology*, Vol. XII, Academic Press, New York and London, 1967, p. 323.
- 6 H. G. Gassen, *J. Chromatogr.*, **39** (1969) 147.
- 7 G. W. Rushizky and C. A. Knight, *Virology*, **11** (1960) 236.
- 8 E. M. Southern and A. R. Mitchell, *Biochem. J.*, **123** (1971) 613.
- 9 B. E. Griffin, *FEDS Lett.*, **15** (1971) 165.
- 10 K. W. Mundry and H. Pries, *Virology*, **46** (1971) 86.

RNA POLYMERASE ACTIVITIES IN THE CYTOPLASM OF DIFFERENTIATING CHICK MUSCLE CELLS

EUGENE BELL AND JOHN BROWN

Department of Biology, Massachusetts Institute of Technology, Cambridge, Mass. 02139 (U.S.A.)

(Received November 29th, 1971)

SUMMARY

Three distinct activities which catalyze the incorporation of radioactivity from [³H]UTP into acid-precipitable material have been recovered from the cytoplasm of embryonic chick muscle cells. On the basis of an analysis of their *in vitro* products, one of them is due to an enzyme which adds terminally to RNA; however, two others are DNA-dependent and synthesize RNA heteropolymers. One of the latter two is recovered cosedimenting with polyribosomes.

These activities have properties distinct from nuclear DNA-dependent RNA polymerases described by others and from nuclear RNA polymerases found in chick embryonic muscle cells, and consequently it seems probable that they function in the cytoplasm *in vivo*. Since the activity found cosedimenting with polyribosomes is sensitive to α -amanitin, it is unlikely to be of mitochondrial origin.

INTRODUCTION

RNA polymerases are known to occur in a variety of adult and embryonic cells (Furth and Loh¹, Widnell and Tata², Jacob *et al.*^{3,4}, Roeder and Rutter^{5,6}, Lindell *et al.*⁷, Tsai *et al.*⁸, Weiss⁹) but little has been reported on their intracellular distribution. Finding RNA polymerase activities in cytoplasmic extracts may mean that the enzymes are en route to the nucleus after having been synthesized on polyribosomal complexes in the cytoplasm or that they have leaked out of the nucleus during preparation of cell fractions or that they actually function in the cytoplasm. In the present paper we describe three distinct activities recovered from the cytoplasm of chick embryonic muscle cells which catalyze the incorporation of radioactivity from [³H]UTP into acid-precipitable material but which differ from nuclear RNA polymerase activities. Two of the activities are DNA dependent while the third is an RNA-terminating enzyme. One of the former cosediments with polyribosomes and is not mitochondrial. These activities have properties distinct from nuclear DNA-dependent RNA polymerases described by others (Jacob *et al.*⁴, Roeder and Rutter⁶) and from nuclear RNA polymerases found in chick embryonic muscle cells, and consequently it seems probable that they function in the cytoplasm *in vivo*.

- 11 J. E. Dahlberg, *Nature*, 220 (1968) 548.
- 12 L. M. Adam, P. G. N. Jeppesen, F. Saenger and B. G. Barrell, *Nature*, 223 (1969) 1009.
- 13 G. W. Rushizky, J. H. Mozejko, D. L. Rogerson, Jr and H. A. Sober, *Biochemistry*, 9 (1970) 4966.
- 14 R. T. Haviland and L. L. Bieber, *Anal. Biochem.*, 33 (1970) 323.
- 15 M. Hiramatsu, T. Uchida and F. Egami, *Anal. Biochem.*, 17 (1966) 135.
- 16 R. F. Beers, Jr, *J. Biol. Chem.*, 235 (1960) 2393.
- 17 D. A. Lloyd and S. Mandel, *Biochemistry*, 9 (1970) 932.
- 18 M. Uziel, Ch. K. Koh and W. E. Cohn, *Anal. Biochem.*, 25 (1968) 77.
- 19 M. Carrara and G. Bernardi, *Biochim. Biophys. Acta*, 155 (1968) 1.

Biochim. Biophys. Acta, 269 (1972) 225-236

, 34 (1970).
(1961).
, 1971).

71 (1970).

d Kleinig, H. J. C.

(1968).
56, 154 (1969).
ers 16, 233 (1971).

A FLUORESCENT NUCLEOSIDE FROM
GLUTAMIC ACID tRNA OF ESCHERICHIA COLI K 12

K. O. Munniger and S. H. Chang

Department of Biochemistry

Louisiana State University

Baton Rouge, Louisiana

Received January 31, 1972

Abstract

Glutamic acid tRNA from E. coli K 12 contains three minor nucleosides in the anticodon loop, namely, 2-methyladenosine, 5-methylaminomethyl-2-thiouridine and a modified pyrimidine nucleoside, which is highly fluorescent. This tRNA has a chain length of 76 nucleotides and does not contain 7-methylguanosine or dihydrouridine as do the other nine sequenced E. coli tRNAs of chain length 76-77 nucleotides.

In this communication we report the finding of an unusual nucleoside in glutamic acid tRNA from Escherichia coli K 12. E. coli K 12 tRNA^{glu}, purified by reverse phase chromatography, was the kind gift of Dr. A. D. Kelmers of Oak Ridge National Laboratory. The tRNA^{glu} was digested with pancreatic and T₁ ribonucleases and the nucleotide sequences of the fragments were determined by established procedures (1). The elution patterns of the pancreatic and T₁ RNase digestions are shown in Figure 1. The oligonucleotides obtained by enzymatic degradation of glutamic acid tRNA are given in Table 1. A preliminary report of the nucleotide sequence of this tRNA has been presented (2, 3) and is shown in Figure 2.

Ohashi, et al. (4) have reported the sequence of the T₁ anticodon fragment (Peak 11a) as CCCUNUCA^{2m}GG and identified N as 5-methylaminomethyl-2-thiouridine. We have digested this fragment with pancreatic RNase and obtained the following result: C, 4 moles; U, 1 mole; A^{2m}C, 1 mole; G, 1

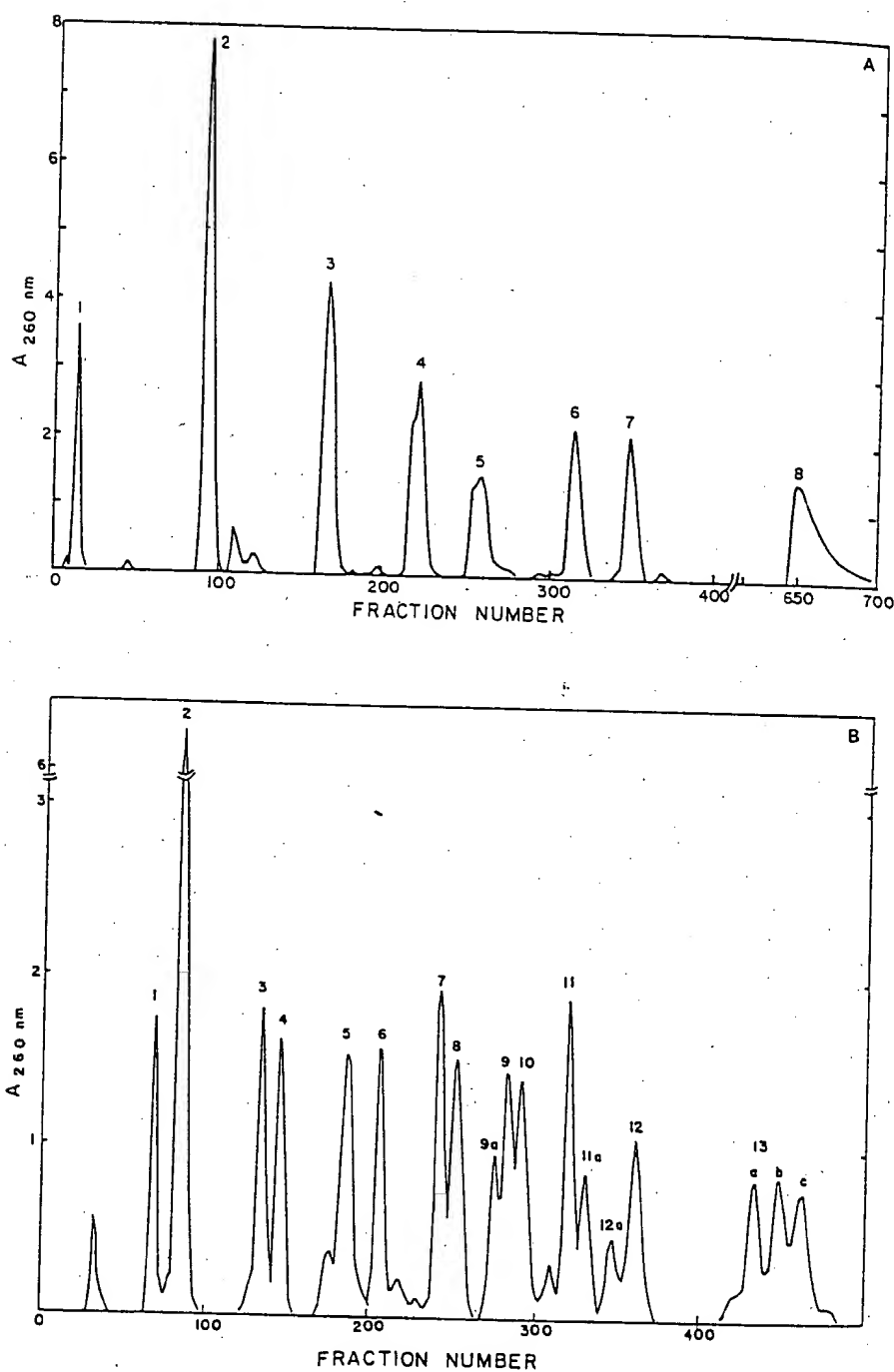


Figure 1 Chromatographic elution patterns of the pancreatic RNase (A) and T₁ RNase (B) digestions of tRNA^{glu}. 550 A_{260nm} units of tRNA was digested with 1.5 mg pancreatic RNase (A) or 3500 units T₁ RNase in a volume of 3 ml containing 30mM trisCl pH 7.5 for 36 hours at 37° and applied to a 0.8x90 cm DEAE cellulose column and eluted with a linear salt gradient, 0.0 to 0.45M NaCl in 7M urea, 20mM trisCl pH 7.5, total volume 2.4 liters.

Table 1. Products Formed by Complete Degradation of Glutamic Acid tRNA with

Pancreatic RNase

Peak 1 A_{OH}Peak 2 18Cp
4Up
2ψpPeak 3 A^{2m}Cp
ACp
2GCp
GUp
NUpPeak 4 AACp
GGCp
GGUpPeak 5 pGUp
GAAUp

Peak 6 AGGACp

Peak 7 AGAGGGCp

Peak 8 AGGGGTp
AGGGGACpT₁ RNasePeak 1 CCA_{OH}

Peak 2 10Gp

Peak 3 CGp

Peak 4 AGp

Peak 5 pGp
ACGp

Peak 6 TψCGp

Peak 7 UCψAGp

Peak 8 CCCAGp

Peak 9a related to Peak 11a

Peak 9 UAACAGp

Peak 10 ACACCGp

Peak 11 UCCCCUUGp

Peak 11a CCCUNFCA^{2m}CGp or
CCCFNUCA^{2m}CGp (i)

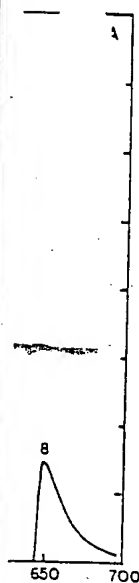
Peak 12a trace, not identified

Peak 12 AAUCCCCUAGp

Peak 13a, b, c, partial fragments

- (i) A paper has appeared (4) giving the sequence of this fragment and identifying N as 5-methylaminomethyl-2-thiouridine, but making no mention of a fluorescent nucleoside, F. The exact position of the nucleoside F in this fragment has not been determined unambiguously.

mole; N, 1 mole; and 1 mole of a highly fluorescent nucleoside, F, the UV absorption spectra of which are shown in Figure 3. F does not absorb appreciably at 260 nm, but at neutral pH the nucleoside has an e_{\max} at 294 nm. Its susceptibility to pancreatic RNase indicates that it is a modified



eatric RNase (A)
A_{260nm} units of
e (A) or 3500
30mM trisCl pH
90 cm DEAE
t gradient, 0.0
total volume

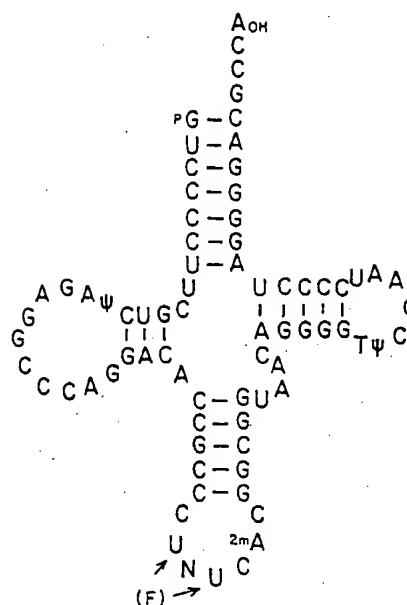


Figure 2 Cloverleaf model of the nucleotide sequence of glutamic acid tRNA of *E. coli* K12. Abbreviations: A, C, G, U: adenylic, cytidylic, guanylic and uridylic acids, respectively; A^{2m}, 2-methyladenylic acid; F, N, see text; ψ, pseudouridylic acid; T, ribothymidylic acid.

pyrimidine. In the pancreatic RNase digestion F_p was not observed probably due to masking by the overwhelming amount of other mononucleotides.

The nucleoside F is very hydrophobic and moves almost with the solvent front in both an isopropanol-water-ammonia (70:20:10 V/V) and an isobutyric acid-water-ammonia (66:33:1, V/V) solvent system. After prolonged treatment in either solvent system the nucleoside remains fluorescent, but slight changes in the UV absorption spectra and apparent. We have not yet determined unambiguously the position of this fluorescent nucleoside in the anticodon loop.

Also notable is the fact that this tRNA does not contain 7-methylguanosine and dihydrouridine, two nucleosides found in all the nine short chained (76-77 nucleotides long) *E. coli* tRNAs sequenced thus far, and the fact that the molecule is resistant to complete digestion by T₁

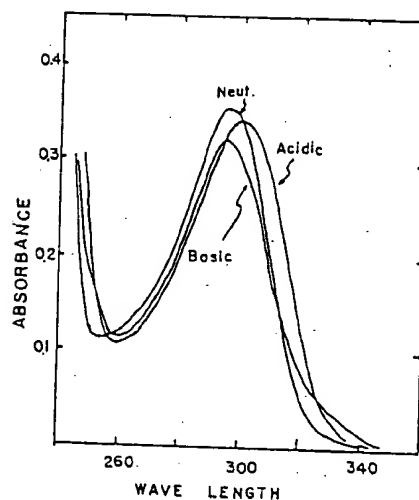


Figure 3 UV absorption spectra of F at neutral, acidic, and alkaline pH.

quence of glutamic acid
A, C, G, U: adenylic,
s, respectively; A^{2m}
t; pseudouridylic

was not observed probably

mononucleotides.

almost with the solvent

10 V/V) and an isobutyric

After prolonged treatment

uorescent, but slight

We have not yet de-

rescent nucleoside in the

not contain 7-

es found in all the nine

RNAs sequenced thus far.

complete digestion by T₁

RNase. This tRNA could not be fully digested with T₁ RNase even under conditions of high enzyme concentration or when supplemented with another guanylohydrolase, N₁ RNase. This resistance to T₁ RNase cannot be solely ascribed to the high number (16 of 20) of GC base pairs conferring a tight tertiary structure on the molecule since Holley (5) completely digested tRNA^{ala} of yeast containing 17 GC base pairs with comparative ease. Whether the absence of 7-methylguanosine or dihydrouridine permits the molecule to assume an unusually rigid tertiary conformation remains to be determined.

Acknowledgements

We thank Dr. A. D. Kelmers of Oak Ridge National Laboratory for supplying us with the purified tRNA^{glu}. This research is partially supported by the National Science Foundation Grant No. GB 17124.

References

1. Rajbhandary, U. L., Faulkner, R. D., and Stuart, A., J. Biol. Chem. **243**, 575 (1968).
2. Munniger, K. O. and Chang, S. H., ACS Southwest Regional Meeting, San Antonio, December, 1971.

3. Munninger, K. O. and Chang, S. H., FEBS Symposium on tRNA, Strasbourg, France, December, 1971.
4. Ohashi, Z., Saneyoshi, M., Harada, F., Hara, H., and Nishimura, S., Biochem. Biophys. Res. Commun. 40, 866 (1970).
5. Holley, R. W., Everett, G. A., Madison, J. T., and Zamir, A. J., J. Biol. Chem. 240, 2122 (1965).

LIBRARY

Acta endocrinologica

Supplementum 168

KAROLINSKA SYMPOSIA
ON RESEARCH METHODS IN REPRODUCTIVE ENDOCRINOLOGY

Organized by Karolinska Institute, Stockholm
with the collaboration of the World Health Organization
and with a grant from the Ford Foundation

New York

Gene Transcription in Reproductive Tissue

Transactions of the fifth Symposium

held in Stockholm

on May 29-31, 1972

EDITOR

F. Diczfalusy

EDITORIAL ASSISTANT

F. Diczfalusy

UNIVERSITY OF CALIFORNIA
CALIFORNIA COLLEGE OF MEDICINE

LIBRARY

175 3 172

IRVINE, CALIFORNIA 92662

PERIODICAL COPENHAGEN 1972

Accompanies Vol. 71

ACEDAB 168 1-453 (1972)

Departments of Microbiology, Obstetrics and Gynecology
and Human Genetics and Development, Columbia University,
College of Physicians and Surgeons New York,
New York 10032, U.S.A.

NUCLEIC ACID-REACTIVE ANTIBODIES SPECIFIC FOR NUCLEOSIDES AND NUCLEOTIDES

By

B. F. Erlanger, D. Senitzer, O. J. Miller
and S. M. Beiser

ABSTRACT

A method is described for making anti-purine and anti-pyrimidine antibodies by immunization with conjugates of naturally-occurring nucleosides or nucleotides with carrier proteins. The specificities of the antisera are presented and shown to be predominantly, if not solely, for the determinant purine or pyrimidine group presented in the antigen. The antisera react with single-stranded or denatured DNA and, in the case of anti-adenosine, with RNA preparations. The antisera have been utilized in biochemical systems to inhibit the priming ability of DNA in a DNA-dependent DNA polymerase system, to detect minor bases such as 6-methyladenosine in DNA, and to detect small areas of single-strandedness in otherwise native DNA. The sera have also been shown to react with living cells, inhibiting the development of fertilized sea urchin eggs and entering and inhibiting transformed Chinese hamster lung cells without affecting normal ones. They have been used in immunofluorescence experiments in which they have been shown to react with nuclear DNA of fixed mouse L-cells harvested during the S phase. Moreover, they have found application in the characterization and identification of human and mouse chromosomes. Finally, initial studies have shown that they can be used in highly specific radioimmunoassays for purine and pyrimidine nucleosides.

The utility of immunological procedures in the study of biological systems is well appreciated. Their high specificity makes them ideally suited for use in analytical procedures, for the localization of components of cells, tissues or organelles, for the modification of activities of specific components in biological systems, and in differentiating among structurally related macromolecules, in particular polysaccharides and proteins. One need go no further than the Karolinska Symposia of 1969 and 1970 to be impressed by the influence of immunology on reproductive physiology, in particular with respect to radioimmunoassay of steroids and gonadotrophins.

The application of immunochemical techniques to problems in genetics and transcription necessarily requires the availability of DNA-reactive antibody. Until as recently as 1957 there was no convincing evidence of the existence of antibody to nucleic acids. At that time, three laboratories reported almost simultaneously the existence of a DNA-reactive antibody-like substance in sera of some patients with systemic lupus erythematosus (Ceppellini *et al.* 1957; Robbins *et al.* 1957; Seligmann 1957; Seligmann & Milgrom 1957). Three years later, Levine *et al.* (1960) reported the first experimental elicitation of DNA-specific antibodies by the immunization of rabbits with lysates of T-even bacteriophage. However, their antibody was specific only for the glycosylated hydroxymethyl cytosine residue unique to T-even phage DNA; no reaction with other DNA preparations could be demonstrated.

During the latter part of the 1950's, our laboratories had begun a search for methods of stimulating anti-DNA antibody. Because of our experience with anti-steroid antibodies (cf. Lieberman *et al.* 1959), the problem was approached by preparing hapten-protein conjugates in which the determinant groups were purine and pyrimidine derivatives. Several such conjugates were prepared, but they did not elicit DNA-reactive antibodies. Bendich and Cohen, aware of our interest, then called our attention to a compound they had synthesized, 6-trichloromethyl purine, which was reactive with amino groups (Cohen *et al.* 1962). This compound could be made to react with the lysine residues of bovine serum albumin (BSA) to yield a conjugate containing 25 purine molecules. The conjugate was immunogenic and yielded purine-specific antibodies that reacted with denatured or single stranded DNA, but not with native DNA. Included were DNA preparations from *E. coli*, *B. subtilis*, *B. natto*, *H. influenza*, chick embryo, calf thymus, T-even phages and ϕ X-174 (Butler *et al.* 1962). Subsequent experiments yielded pyrimidine-specific antibodies which were elicited by immunization with an acetyluracil-protein conjugate (Tanenbaum & Beiser 1963).

We had shown, therefore, that it was possible to raise DNA-reactive antibodies experimentally by using suitable hapten-protein conjugates. In order to do so, however, it was necessary to synthesize reactive purine or pyrimidine analogues that could be conjugated with protein. Ideally, one would want to

be able to utilize naturally occurring nucleosides or nucleotides and, therefore, considerable effort was exerted to find such a method. In 1964, we reported a successful technique (Erlanger & Beiser 1964). The starting materials (Fig. 1) were ribonucleosides or ribonucleotides. (The analogous 2'-deoxy compounds cannot be used.) They were allowed to react at room temperature with a slight excess of periodate which oxidized the vicinal 2' and 3' hydroxyl groups to aldehyde groups. The aldehydic derivatives were coupled to BSA at pH 9-9.5 via the formation of an addition product with the lysine amino groups of the protein. This product was then stabilized by reduction with NaBH_4 to the tertiary amine.

The steps described in Fig. 1 were carried out sequentially without the isolation of any of the intermediate products. It is thus an extremely simple procedure which can effect the conjugation of as many as 30 haptenic groups per molecule of BSA. Moreover, it is applicable to the conjugation of any haptenic compound having vicinal hydroxyl groups and has been used by others to conjugate the cardiac glycoside, digoxin, to a protein carrier for the elicitation of digoxin-specific antibodies (Butler *et al.* 1966; Butler & Chen 1967). Presumably, glycosides or glucuronides of steroids could be handled in the same way. Shown in Table 1 are the various nucleoside and nucleotide determinants to which antibody has been produced in our laboratories. The determinant groups include the five major purine and pyrimidine bases, some so-called minor bases, and dinucleotide phosphates.

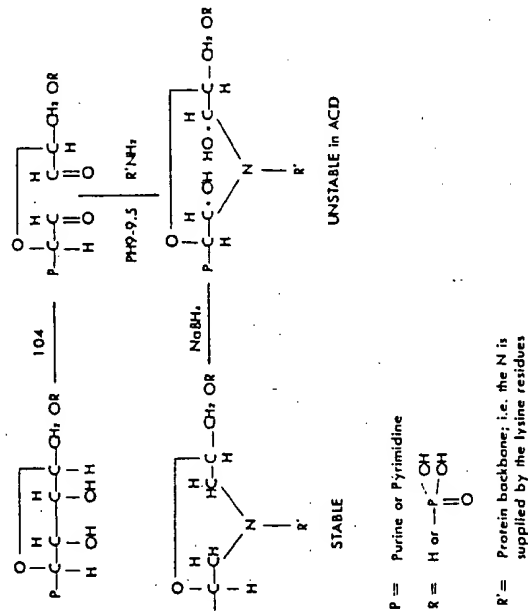


Fig. 1.

Preparation of nucleoside-protein conjugates.

Table 1.
Anti-nucleoside and anti-nucleotide antibodies.

Determinant group	Reference(s)
adenosine	Klein <i>et al.</i> (1966)
adenosine 5'-phosphate	Erlanger & Beiser (1964)
adenosine triphosphate	Erlanger & Beiser, unpublished
uridine	Klein <i>et al.</i> (1967)
uridine 5'-phosphate	Erlanger & Beiser (1964)
guanosine	Erlanger & Beiser (1964)
cytidine	Erlanger & Beiser (1964)
thymidine (5-methyluridine)	Erlanger & Beiser (1964)
NAD	Erlanger & Beiser (1964)
6-methyladenosine	Erlanger & Beiser, unpublished
5-iodouridine	Sawicki <i>et al.</i> (1971)
5-bromouridine	Sawicki <i>et al.</i> (1971)
adenyllyl cytidine (3'-5')	Nahon, unpublished, cited in Beiser & Erlanger (1966)
adenyllyl adenosine (3'-5')	Nahon, unpublished, cited in Beiser & Erlanger (1966)
adenyllyl uridine (3'-5')	Nahon, unpublished, cited in Beiser & Erlanger (1966)
cytidyllyl adenosine (3'-5')	Nahon, unpublished, cited in Beiser & Erlanger (1966)
uridyllyl adenosine (3'-5')	Nahon, unpublished, cited in Beiser & Erlanger (1966)
cytidyllyl cytidine (3'-5')	Nahon, unpublished, cited in Beiser & Erlanger (1966)
cytidyllyl guanosine (3'-5')	Nahon, unpublished, cited in Beiser & Erlanger (1966)
guanylyl cytidine (3'-5')	Seitzner, unpublished
adenyllyl guanosine (3'-5')	Seitzner, unpublished
guanylyl adenosine (3'-5')	Wallace <i>et al.</i> (1971)
	Wallace <i>et al.</i> (1971)

A number of the antisera have been purified by specific adsorption to egg albumin conjugates followed by elution by hapten or other means (Szafran *et al.* 1969).

Specificity of the antisera

The reaction of antigen with antibody and with denatured DNA has been measured by complement fixation, microquantitative precipitation, agar gel diffusion and the double antibody technique. The specificities of the antibodies have been delineated further by hapten inhibition of precipitin or of complement fixation. The antisera are highly specific. Unlike our results with anti-steroid antibodies (Beiser *et al.* 1959), among which considerable cross reaction occurred, the anti-purine and anti-pyrimidine antisera, in general, exhibited very little cross reaction. It is not unusual to find antisera showing reaction with the immunizing antigen only. An example is shown in Fig. 2, in which is presented a curve of the complement fixation reaction between a globulin pre-

paration of an anticytidine serum and various purine and pyrimidine conjugates, as well as BSA (Garro *et al.* 1968). In this case, the specificity of the serum was absolute; no cross reactions were observable. Other anti-cytidine sera, obtained at the same time, showed cross reaction with guanosine-BSA but with no other conjugate. Absorption of the latter sera with guanosine-BSA yielded antisera specific for the cytidine determinant group only.*

Thus, by using the »periodate procedure« described above, it is possible to obtain essentially monospecific anti-purine or anti-pyrimidine sera, either directly from the animal or after appropriate absorption procedures.

As with the homologous purine- or pyrimidine-protein conjugates, reaction with denatured DNA could be demonstrated by complement fixation, precipitation, gel diffusion and double antibody techniques. Precipitation reaction frequently required that DNA be denatured by heat in the presence of formaldehyde although this was not true for all types of antisera. Inhibition by hapten was always greatest by far with the hapten used as the determinant group in the antigen.

Numerous attempts to demonstrate a reaction between RNA and anti-nucleoside antisera by precipitation or by complement-fixation were negative. Recently, we were able to detect a reaction with labelled RNA using the double antibody technique in the presence of 0.2 M Na_2SO_4 , which inhibits RNase. Of interest, and as yet unexplained, was the finding that of the anti-mono-nucleoside sera tested, only anti-adenosine reacted with RNA. Of the RNA preparations tested, all but t-RNA reacted with anti-adenosine, but, even after denaturation, none of the RNA preparations reacted with antisera having other than adenosine specificity (Rosenberg 1970, 1971).

* It is interesting to note that, in general, an examination of the chemical structure of a determinant group can lead one to predict the types of cross reactions that will occur with a particular antibody. Thus, for example, in the case of the anti-steroid antibodies, it was not unreasonable to find cross reactions among the adrenocortical hormones and the androgens, since they share a very similar polycyclic nucleus (Beiser *et al.* 1959). This type of prediction, however, cannot be made with the anti-purine or anti-pyrimidine antisera. For example, one might have supposed that anti-guanosine antibodies would cross-react with adenosine, at least to some extent. This has never been observed nor have we observed cross reactions among the pyrimidines. It appears, therefore, that purines and pyrimidines have special properties *vis-à-vis* biological recognition systems, such that there is no ambiguity in their recognition. This attribute is probably unique to these classes of compounds and is carried over into other recognition systems, such as those involved in replication and in transcription. Put in another way, we are looking at another example of how purines and pyrimidines can convey exact information.

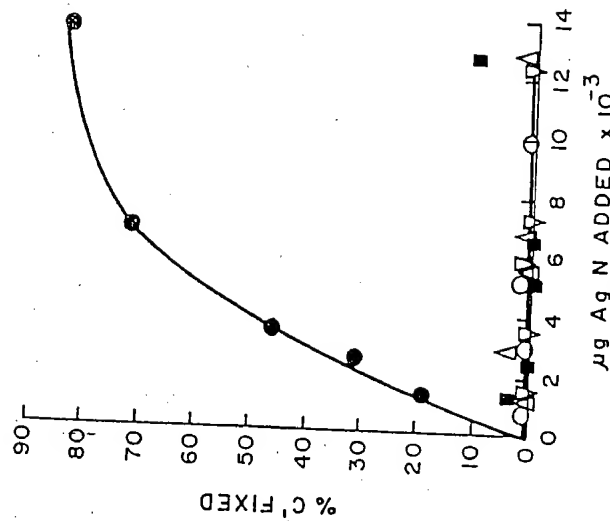


Fig. 2.

Complement fixation reactions between nucleoside-BSA conjugates and anti-cytidine-BSA globulin fraction. ● cytidine-BSA; ■ thymidine-BSA; △ adenosine-BSA; ○ guanosine-BSA; ▽ BSA.

UTILIZATION OF ANTI-PURINE AND ANTI-PYRIMIDINE ANTIBODIES

In biochemical systems

Anti-guanosine and anti-thymidine antibodies (the latter prepared by immunizing with a BSA conjugate of 5-methyl uridine) were capable of affecting the ability of denatured calf thymus DNA to act as a primer for a DNA polymerase extracted from chick embryos (Wallace *et al.* 1969). A decrease in rate of incorporation of tritiated thymidine was observed but, more significantly, the relative kinetics of tritiated thymidine incorporation in the presence and absence of antibody resembled those of chromatin and DNA isolated from chromatin in a DNA-dependent RNA polymerase system (Marushige & Bonner 1966). In other words, it appeared that the antibody mimicked the action of chromatin, i. e. it was masking portions of the DNA, making these portions unavailable for copying.

Recently, Sawicki *et al.* (1971) demonstrated that anti-nucleoside antisera could be used to detect so-called minor bases in DNA preparations. Antisera to 6-methyladenosine were so highly specific that negligible cross reaction

occurred with adenosine. These antisera could detect as little as 1% 6-methyladenosine in a DNA preparation.

A. Garro, in our laboratory, was able to show that anti-purine and anti-pyrimidine sera could be used to detect small areas of single strandedness in otherwise native DNA (Garro *et al.* 1968). Utilizing a photooxidation process specific for the destruction of guanine, it was demonstrated that, although native DNA was unreactive, reaction with anti-cytosine became detectable and increased as guanosine residues were oxidized. No reaction with anti-thymidine was observed, confirming that destruction of individual guanine residues exposed only the formerly paired cytosine residues to anti-cytidine. The data indicate that destruction of less than 10% of the guanosine could be detected

Reactions in living cells

In early studies, Rosenkranz *et al.* (1964) demonstrated that anti-purine and anti-pyrimidine antibodies could penetrate fertilized sea urchin eggs and affect the development of the embryos. More recently, Dr. Liebeskind, in our laboratories, found that anti-thymidine antisera entered transformed Chinese hamster lung cells and inhibited their growth (Liebeskind *et al.* 1971). Normal globulin also entered the transformed cells but did not inhibit growth. On the other hand, normal Chinese hamster lung cells were penetrated neither by anti-thymidine nor by normal globulin and hence were unaffected. We are continuing these studies with other cell lines and antisera since they may be of practical therapeutic significance.

Immunofluorescence experiments

Klein *et al.* (1967) showed that fluoresceinated anti-nucleoside globulin would react with the nuclei of fixed mouse L-cells (Fig. 3) but only if these cells were harvested during the period of maximal DNA synthesis as measured by uptake of thymidine. This corresponded to the time when DNA was replicating (the S phase) and was at least partially single-stranded. Freeman *et al.* (1971) pursued these studies further with human diploid fibroblasts, examining the nuclear fluorescent properties throughout the entire cell cycle. Their results confirmed Klein's. Moreover, strikingly different patterns were observed during the early, middle and late portions of the S phase. In particular, nucleolar fluorescence occurred very early during the S phase. This was followed by membrane fluorescence and finally by fluorescence within the nucleus.

One of the recent applications of anti-nucleoside antibodies is the investigation of chromosome structure. Standard 3:1 methanol: acetic acid fixed human or mouse metaphase chromosomes were examined. For reaction with antibody

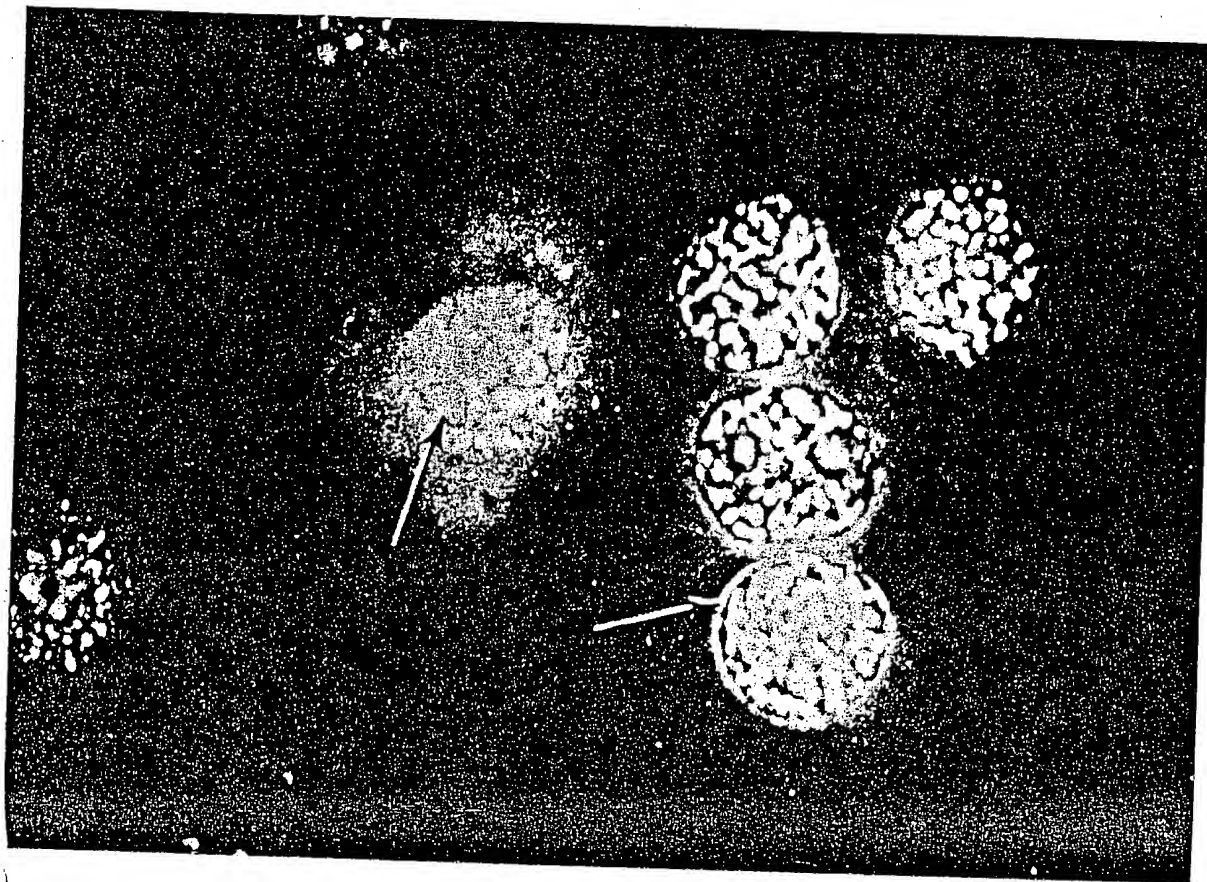


Fig. 3.

Fluorescence of L cell nuclei using fluorescein-conjugated sheep anti-adenosine. Arrows point to a nucleolus and to a nuclear membrane. (Klein *et al.* 1967) (Reprinted with permission of Rockefeller University Press).

it was necessary to denature the DNA (Freeman *et al.* 1971) and this was done by a one-half to one hour treatment at 65° with 95% formamide in 0.15 M NaCl - 0.015 M sodium citrate (SSC), with or without 0.25% formaldehyde. Using the indirect immunofluorescent method, anti-adenosine, anti-guanosine, anti-thymidine and anti-cytidine produced a series of light and dark banded chromosomes (Fig. 4). The fluorescent banding followed a consistent pattern that resembled the banding pattern of quinacrine-stained human chromosomes (Caspersson *et al.* 1970) and the Giemsa banding seen after pretreatment with trypsin (Seabright 1971) or hot SSC (Sumner *et al.* 1971).



Fig. 4. Karyotype of a cell from normal human male, treated as described in text with rabbit anti-adenosine and fluorescein-tagged sheep anti-rabbit globulin. Zeiss fluorescent microscope, HBO 200 W high pressure lamp, BG12 exciter and 530 nm barrier filter.

After treatment with anti-adenosine, each chromosome in the metaphase cell could be identified by its characteristic banding pattern (Fig. 4). Despite the similarity to the other banding patterns, there are certain characteristic features of the anti-nucleoside-treated chromosomes. The non-staining region at the centromeres is more prominent and the non-staining secondary constriction regions of chromosomes 1, 9 and 16 are also larger. The distal end of the Y, which is intensely fluorescent after quinacrine staining, is almost unreactive with anti-adenosine. Preliminary investigations indicate that anti-adenosine and anti-thymidine produce more distinctive patterns than the other antisera. It is interesting to note that, after trypsin or hot SSC treatment, which are used to bring out Giemsa-banding, no anti-adenosine is taken up by the chromosomes. Differential denaturation or renaturation cannot, therefore, be responsible for the Giemsa-banding patterns, as has been suggested by Sumner *et al.* (1971).

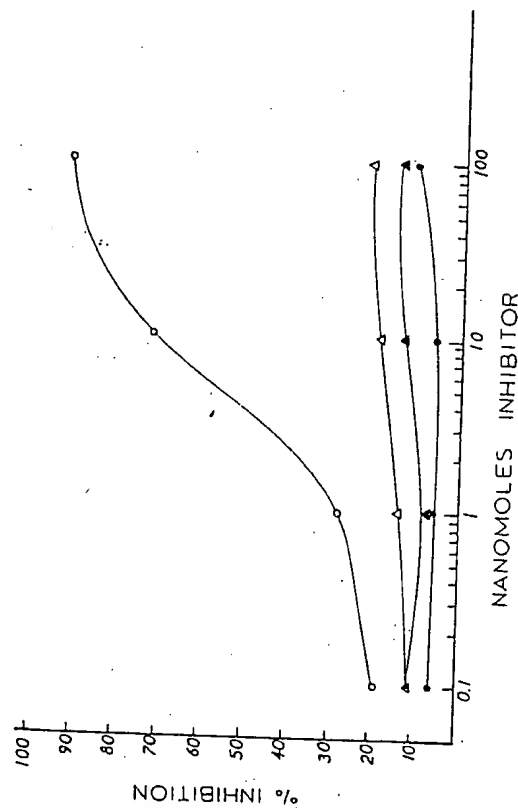


Fig. 5. Hapten-inhibition of binding of heat-denatured, tritiated *E. coli* DNA by various nucleosides. ● adenosine; ○ cytosine; ▲ guanosine; △ thymidine. Conditions of experiment: The anti-cytidine dilution was such that 40% of DNA was bound in absence of hapten. Litter was incubated with 50 λ antibody for one hour at 4° in 0.01 M Tris, 0.15 M NaCl, pH 7.4 (TBS). Then tritiated DNA was added and the mixture kept at 4° overnight. A quantity of sheep anti-rabbit globulin was added which was 20% more than required to precipitate all anti-cytidine. After remaining overnight at 4°, the precipitate was collected by centrifugation, washed 3 times with TBS, dissolved in Soluene (Packard Instruments) and counted in 10 ml Omnifluor (New England Nuclear).

Radioimmunoassay

Like other anti-hapten antisera, anti-nucleoside sera should be suitable for use in radioimmunoassays of free nucleosides. A beginning has been made in our laboratories using a somewhat novel approach: measurement of the displacement of tritiated DNA by «cold» nucleoside. As shown in Fig. 5, the assay is extremely specific; displacement of DNA from anti-cytidine is accomplished by cytidine at very low concentrations. The other bases are, for practical purposes, inactive. The same kind of specificity is shown by sera specific for the other purine and pyrimidine bases.

CONCLUSION

The importance of the tools and techniques of immunology in biological studies has become increasingly recognized within the last decade. In fact, very few fields of biology have not felt the impact of immunology. The development of means of producing nucleic acid-reactive antibodies specific for purine and pyrimidine bases, makes it possible now to expand the utilization of immunological techniques into fields hitherto untouched. Most obvious among these fields is genetics; in many respects, however, the problems of genetics and those of reproductive physiology overlap and we can safely predict that nucleic acid-reactive antibodies can be utilized in these areas. There certainly is precedent for saying that the development of new tools usually leads to their application in ways unforeseen by those involved in their development. The authors hope that the presentation of our material at this interdisciplinary symposium will lead to unanticipated, original applications to problems in reproductive physiology.

ACKNOWLEDGMENTS

We wish to acknowledge the support of the National Institutes of Health (Grants CA 12504, GM-18153 and AI-00860), the Office of Naval Research (Contracts 266(40) and 4259(11)) and the National Foundation March of Dimes.

REFERENCES

- Beiser S. M. & Erlanger B. F.: *Cancer Research*. 26 (1966) 2012.
- Beiser S. M., Erlanger B. F., Agate F. J. & Lieberman S.: *Science* 129 (1959) 564.
- Bulter V. P. Jr. & Chen J. P.: *Proc. Nat. Acad. Sci. U.S.* 57 (1967) 71.
- Bulter V. P. Jr., Beiser S. M., Erlanger B. F., Tanenbaum S. W., Cohen S. & Bendish A.: *Proc. Nat. Acad. Sci. U.S.* 48 (1962) 1597.

- Bulter V. P. Jr., Erlanger B. F. & Chen J.: *J. clin. Invest.* 45 (1966) 993.
- Casperston T., Zech L. & Johanson C.: *Exp. Cell Res.* 62 (1970) 490.
- Celipetimi R., Polli E. & Gelada F.: *Proc. Soc. exp. Biol. (N.Y.)* 96 (1957) 572.
- Cohen S., Thom E. & Bendish A.: *J. org. Chem.* 27 (1962) 3545.
- Erlanger B. F. & Beiser S. M.: *Proc. Nat. Acad. Sci. U.S.* 52 (1964) 68.
- Freeman M. V. R., Beiser S. M., Erlanger B. F. & Miller O. J.: *Exp. Cell Res.* 69 (1971) 345.
- Garro A. G., Erlanger B. F. & Beiser S. M. In: Plescia O. J. and Braun W., Eds. *Nucleic Acids in Immunology*. Springer-Verlag: New York (1968) 47.
- Klein W. J. Jr., Beiser S. M. & Erlanger B. F.: *Bact. Proc.* (1966) 56.
- Klein W. J. Jr., Beiser S. M. & Erlanger B. F.: *J. exp. Med.* 125 (1967) 61.
- Levine L., Murakami W. T., Van Vinakis H. & Grossman L.: *Proc. Nat. Acad. Sci. U.S.* 46 (1960) 1038.
- Lieberman S., Erlanger B. F., Beiser S. M. & Agate F. J.: *Recent Progr. Hormone Res.* 15 (1959) 165.
- Lieskind D. S. P., Hsu K. C., Erlanger B. F. & Beiser S. M.: *Nature (Lond.)* 234 (1971) 127.
- Marushige K. & Bonner J.: *J. molec. Biol.* 15 (1966) 160.
- Robbins W. C., Holman R. H., Deicher H. R. G. & Kunkel H. G.: *Proc. Soc. exp. Biol. (N.Y.)* 96 (1957) 575.
- Rosenberg B. J.: *Fed. Proc.* 29 (1970) 829.
- Rosenberg B. J.: Ph.D. Thesis (1971) Columbia University, New York.
- Rosenkrantz H. S., Erlanger B. F., Tanenbaum S. W. & Beiser S. M.: *Science* 145 (1964) 282.
- Sawicki D. L., Erlanger B. F. & Beiser S. M.: *Science* 174: (1971) 70.
- Seabright M.: *Lancet* ii (1971) 971.
- Stegmann M.: *Compt. Rend.* 245 (1957) 243.
- Stegmann M. & Milgrom F.: *Compt. Rend.* 245 (1957) 1472.
- Sumner A. T., Evans H. J. & Buchland R. A.: *Nature New Biol.* 232 (1971) 31.
- Szafraan H., Beiser S. M. & Erlanger B. F.: *J. Immunol.* 103 (1969) 1157.
- Tanenbaum S. W. & Beiser S. M.: *Proc. Nat. Acad. Sci. U.S.* 49 (1963) 662.
- Wallace S. S., Erlanger B. F. & Beiser S. M.: *J. molec. Biol.* 43 (1969) 41.
- Wallace S. S., Erlanger B. F. & Beiser S. M.: *Biochemistry* 10 (1971) 679.

DISCUSSION

Erlanger: Recent experiments by R. Schreck in our laboratories indicate that chromosomes of human cells in metaphase can be photooxidized by visible light in the presence of methylene blue, as can be done with DNA in solution (Garro *et al.* 1968). Subsequent staining with anti-cytidine sera followed by fluorescein-labelled sheep anti-rabbit sera (indirect procedure) yielded banded chromosomes whose patterns were, in general, complementary to those shown in Fig. 4. A control with anti-thymidine gave no banding. These results are in accord with the earlier findings of Simon & Van Vinakis (1962) that guanine residues are selectively destroyed by methylene blue-sensitized photooxidation and with the evidence of Weisblum & de Hasel (1972) that quinacrine fluorescence occurs in regions of DNA rich in A-T base pairs. It is, therefore, feasible to combine specific chemical and immunochemical techniques to investigate the structure of chromosomes.

Cocito: Supposing you make antibodies against a dinucleotide. Let us say A and C, would the antibodies react with A, C and AC with the same efficiency?

Erlanger: If you make antibody to ApC, let us say, and C is the moiety that is attached to the protein, the antibodies react most with the determinant group of the conjugate used for immunization, that is with ApC. There is also cross reaction with C, but not with A.

Cocito: If you now adsorb your antibody preparation on C, what is left, does it react only with A?

Erlanger: There is reaction only with ApC; none with C, and no reaction with A.

Cocito: What about the trinucleotides?

Erlanger: We are working on that now. The trinucleotides, we hope, will be seen as trinucleotides. We are hoping that we will be making antibody specific for a sequence. But the first problem is to make the trinucleotides in a quantity sufficient for immunization of rabbits; that means making 10-15 milligrams of it.

Cocito: Did you obtain antibodies against methylated albumin bound RNA?

Erlanger: We have not tried to do that.

Dixon: What happens to the rabbits which make these antibodies, have they developed autoimmune problems of any kind?

Erlanger: We have never been able to find it. Obviously, they could develop lupus, for example. We don't have any evidence that any rabbits have come down with lupus as a result of immunization.

Bouteille: We have been developing (*Leduc et al.* 1968) methods of antibody labelling with peroxidase, and our hope, of course, was to label with a high resolution in the nucleus histones and eventually nucleic acids. In that way we would be able, for instance, to distinguish between DNA and RNA. I realize that you have difficulties with RNA. We could overcome that problem by digesting on sections with DNase or RNase just before the reaction. I think this is a possibility and I would like to have your opinion.

Erlanger: I think that in terms of visualizing DNA and RNA complexes with antibody by electron microscopy you can probably succeed with some type of digestion of DNA or RNA and then look for antibody tagged either with peroxidase or ferritin and find it. The procedure that we use would be bothered by digestion, because we would then be producing inhibitors of the reaction, haptenic inhibitors. So we couldn't do this. On the other hand, there might be things we can do that would allow us to get better reactions with RNA. But there is no question about this: under conditions that are identical, in which we get reaction with DNA, we only get reaction of RNA with anti-A.

Caspersson: These applications of immunofluorescence techniques on chromosomes open up really exciting possibilities, and we look forward to seeing more work done with different antibodies, and also comparisons between the resulting patterns. I would like to draw your attention to the TV-based technique for the study of chromosome pattern details which I described in my presentation. That procedure facilitates very

much detailed comparisons between details in patterns produced by different techniques.

Erlanger: I would like to point out with respect to peroxidase labelling that there is a possibility that we'll be able to see something interesting with that type of antibody labelling rather than fluorescent labelling. Fluorescent labelling reminds me of looking into the headlights of an automobile; you can see the headlights but you cannot see the automobile. You can't see the details. It has an advantage in that you can measure it accurately because you are measuring something over background noise rather than measuring difference. But in terms of seeing details, fluorescence has disadvantages, and I think that with peroxidase-labelled antibody we'll be able to see some details that we cannot see with fluorescence. And of course, we will be able to do electron microscopy using the same antiserum.

Caspersson: I am not sure I agree entirely with your looking into the headlights. That depends very much on the technique you use. You have to use rather subtle techniques to pick out all the details.

Pastan: We find that when we make antibodies to nucleotides, such as cyclic AMP and cyclic GMP, then one doesn't end up with such great specificity. This is probably because the chemical method of making the conjugates is different.

The second thing that struck me is your finding that there is no single-stranded DNA in interphase cells. A model has been proposed by *Crick* for control of DNA expression in animal cells in which single-stranded regions of DNA are present in normal cells during interphase. I think your data are the strongest evidence against that proposal.

Erlanger: I wouldn't want to get into battle with *Crick*: But you have to remember that we would be able to find single-stranded regions only if the antibody molecule can get in. This is a molecule of 180 000 molecular weight. So our methods are limited by that fact.

Pastan: The cells are transformed cells; are those the cells you have employed in these experiments?

Erlanger: Dr. Kellin's work was done with mouse L cells. Dr. Liebeskind worked with normal and methylcholanthrene-treated (*i.e.* transformed) Chinese hamster lung cells.

Hubinont: You probably didn't study the species cross reaction possibility?

Erlanger: No. You don't see a species difference as you do with proteins.

Hubinont: I guess that adjuvants added to your pure antigen instead of coupling it with large molecules of bovine albumin cannot be used in these studies because most of these adjuvants contain bacterial material?

Erlanger: Oh, yes, we use adjuvants. Let me make clear first of all that the purine and pyrimidine are attached covalently to the protein. Pyrimidine or purine does not produce the antibody. These are haptens, groups that react with antibody but are not immunogenic. So it becomes a determinant group and immunogen only when it is attached covalently to a carrier. We do use adjuvants. The way in which we immunize is to use adjuvants and to immunize in the foot pad of rabbits. There is no problem with this.

Hubinon: it has no antigenic properties by itself. What happens when you mix your preparation with adjuvant and inject it into the rabbit without coupling to albumin?

Erlanger: The nucleotides will not induce antibodies if mixed with adjuvant and injected into animals.

Mach: About the difficulty of getting antibodies to RNA: It is not what bothers the animal, but what bothers the cell, which might be crucial. It is interesting that the antibodies are very compartmentalized in the cell. If a cell started producing its own cytoplasm antibodies against ribosomes, for instance, the cell would very likely die and stop growing. Antibodies against DNA are probably well tolerated by the cells because the antibody never gets the chance to see the DNA inside the cells.

Since this is a symposium on methods, I just want to mention briefly an immunological chemical procedure which Dr. Ch. Faust and I have developed, not to select DNA but to select specific polysomes and thus specific messenger RNAs. This technique involves the purification on a reversed immunoabsorbent column of polysomes specific for a given protein. The column contains covalently coupled antibodies directed against the immunogenic nascent polypeptide chains. In our case, we have selected for polysomes specific for the L chain of gamma globulin. One can load the columns with polysomes and the column retains a certain number of these polysomes specifically bound by the nascent chain. The next step involves the recovery of the specific polysomes intact. For this the antibiotic puromycin can be used. Puromycin cleaves the nascent chains from the polysomes and releases "nascent chain-free polysomes". Various controls can show these to be specific. We are proposing that this method could be very generally applicable, not only to L chain polysomes, but to other types of polysomes, and therefore to other types of messengers as well.

Erlanger: That is very interesting. I would just like to comment on your first comment and that is to make absolutely clear here that we are not making antibodies to DNA in our procedure. We are making antibodies that cross-react with DNA and we are looking for cross-reactions with RNA.

People have made antibodies to DNA by complexing them to various things, like methylated bovine albumin, for example. People have made antibodies to ribosomes and the fact that it would not be healthy for an animal to make antibodies to ribosomes is not relevant because you can do these things. You can get antibodies just by exposing them to things they will never be exposed to normally and they will frequently make antibodies.

Lewin: I should like to come back to the matter Dr. Pastan raised. Can you say what the resolution of your technique is? How long would a denatured region have to be for you to be able to detect it?

Erlanger: Garro's experiments did not include studies at a very low level of photooxidation. We were able to see reaction of anti-C antibody after 2-3% of the guanosine was destroyed. We don't know where this guanosine was located. We don't know if it was concentrated at one locus, or whether we were destroying residues at random. So I cannot give you a direct answer. I can say that since the structure of antibody molecules is known, you can make an estimate as to how big the grouping would have to be, but we have not made any calculations.

Reference:

- Garro A. G., Erlanger B. F. & Beiser S. M. In: Plescia O. J. and Braun W., Eds. Nucleic Acids in Immunology. Springer Verlag, New York (1968) 47.
Leduc E. H., Avrameas S. & Bouteille M.: J. exp. Med. 127 (1968) 109.
Simon M. I. & Van Vunakis H.: J. molec. Biol. 4 (1962) 488.
Wetsblum B. & de Haseth P. L.: Proc. Nat. Acad. Sci. U.S.A. 69 (1972) 629.

- Kelly, R. B., and Sinsheimer, R. L. (1967), *J. Mol. Biol.* 29, 229.
- Lipsett, M. N. (1960), *Proc. Nat. Acad. Sci. U.S.A.* 46, 445.
- Maxwell, I. H., Wimmer, E., and Tener, G. M. (1968), *Biochemistry* 7, 2629.
- Michelson, A. M., Massoulié, J., and Guschlbauer, W. (1967), *Progr. Nucl. Acid. Res. Mol. Biol.* 6, 83.
- Muench, K. H., and Berg, P. (1966), in *Procedures in Nucleic Acid Research*, Cantoni, G. L., and Davies, D. R., Ed., New York, N. Y., Harper & Row, p. 375.
- Rich, A., Davies, D. R., Crick, F. H. C., and Watson, J. D. (1961), *J. Mol. Biol.* 3, 71.
- Sedat, J. W., Kelly, R. B., and Sinsheimer, R. L. (1967), *J. Mol. Biol.* 26, 537.
- Sedat, J., Lyon, A., and Sinsheimer, R. L. (1969), *J. Mol. Biol.* 44, 415.
- Steiner, R. F., and Beers, Jr., R. F. (1957), *J. Polym. Sci.* 30, 17.
- Steiner, R. F., and Beers, Jr., R. F. (1961), *Polynucleotides*, Amsterdam, Elsevier Publishing Co., p. 232.
- Stern, R., and Cooper, H. L. (1968), *Fed. Proc., Fed. Amer. Soc. Exp. Biol.* 27, 835.
- Stern, R., and Friedman, R. M. (1969), *J. Virol.* 4, 356.
- Straus, Jr., J. H., and Sinsheimer, R. L. (1963), *J. Mol. Biol.* 7, 43.
- Tomlinson, R. V., and Tener, G. M. (1962), *J. Amer. Chem. Soc.* 84, 2644.
- Tomlinson, R. V., and Tener, G. M. (1963), *Biochemistry* 2, 697.

Chemical Modification of the Fluorescent Base in Phenylalanine Transfer Ribonucleic Acid*

Doju Yoshikami† and Elizabeth B. Keller‡

ABSTRACT: The tRNA^{Phe} of wheat germ, tRNA₁^{Phe}, exhibits fluorescence in solution due to the presence of a fluorescent base, Y_w¹, adjacent to the 3' end of the anticodon. When this tRNA was exposed to ammonium carbonate at pH 9, it was converted into tRNA₂^{Phe} which exhibits the same fluorescence but is chromatographically distinct from tRNA₁^{Phe}. The conversion was due to the modification of Y_w¹ to a new fluorescent base, Y_w², which has a free acidic group (pK_a = about 4) not present in Y_w¹. Thus at around neutrality tRNA₂^{Phe} has an acidic group with a net negative charge on the base adjacent to the 3' end of the anticodon; in every other respect it is identical with tRNA₁^{Phe}. The specific modification had no effect upon the rate at which the tRNA was esterified by the Phe-tRNA synthetase, but it reduced the rate of poly(U)-directed polyphenylalanine synthesis. The free base Y_w² can be further

degraded by alkaline hydrolysis to Y_w³ and then to Y_w⁴ without any change in the spectrum of the fluorescent chromophore.

In the conversion of Y_w³ into Y_w⁴ a blocking group is removed from an aliphatic amino group on a side chain. These results indicate that Y_w¹ is similar to the Y base of yeast in having a blocked amino acid side chain on the characteristic Y base chromophore. This paper presents evidence that Y_w¹ differs from the Y base of yeast in the structure of the distal portion of the side chain. The Y base of beef tRNA^{Phe} is indistinguishable from that of wheat germ. A simple procedure was found for purifying wheat germ and yeast tRNA^{Phe} employing two benzoylated DEAE-cellulose columns, one run in the presence of unbuffered MgCl₂ and one in the presence of EDTA buffered at pH 4.5.

The major phenylalanine tRNAs (tRNA^{Phe}s) isolated so far from eukaryotic organisms have all been distinguished by the presence of an unusually hydrophobic and highly fluorescent base, the Y-type base. A base of this type was first detected in the tRNA^{Phe} of yeast by RajBhandary *et al.* (1967) and was called Y. It was found to be located contiguous with the 3' end of the anticodon. A related Y base was found in the same position in the structure of the tRNA^{Phe} of wheat germ

by Dudock *et al.* (1969). A Y-type base is present, presumably in the same location, in the tRNA^{Phe}s from rat liver (Fink *et al.*, 1968), beef liver (Yoshikami *et al.*, 1968), and peas (G. A. Everett, personal communication). The Y base has not been detected in any other species of tRNA other than tRNA^{Phe} (Yoshikami *et al.*, 1968). The Y base thus appears to have a role unique to the function of the tRNA^{Phe}s of eukaryotes, yet it is not an essential feature of tRNA^{Phe} in general since it is not present in the tRNA^{Phe} of *Escherichia coli* (Barrell and Sanger, 1969).

Other tRNAs exhibit, in the same locus adjacent to the 3' end of the anticodon, a wide variety of hypermodified residues (Schweizer *et al.*, 1969) such as 1-methylinosine (Holley *et al.*, 1965), N⁶-isopentenyladenosine (Biemann *et al.*, 1966), N⁶-isopentenyl-2-methylthioadenosine (Burrows *et al.*, 1968), and N-(purin-6-ylcarbonyl)threonine ribonucleoside (Schweizer *et al.*, 1969). It has been found that most of the tRNAs which have an A as the 3' base of the anticodon possess a hyper-

* From the Section of Biochemistry and Molecular Biology, Cornell University, Ithaca, New York 14850. Received January 26, 1971. This work was supported by Public Health Service Research Grant No. GM 10791 and Training Grant No. GM 00824 from the National Institutes of General Medical Sciences. A portion of this work has appeared in preliminary form (Yoshikami and Keller, 1969). Taken in part from a dissertation submitted by D. Y. to Cornell University for the degree of Doctor of Philosophy.

† Present address: Department of Neurobiology, Harvard University Medical School, Boston, Mass. 02115.

‡ To whom correspondence should be addressed.

isopentenyl-2-methylthioadenosine (Nishimura *et al.*, 1969; Peterkofsky and Jesensky, 1969; Rosenberg and Gefter, 1969). To investigate the role of the hypermodified base adjacent to the anticodon, we undertook a study of the tRNA^{Phe} of wheat, beef, and yeast, all of which contain a Y-base in this position. In the course of the study of the tRNA^{Phe} of wheat germ it was found that the Y-base can be chemically modified *in situ* under conditions sufficiently mild that other bases in the tRNA remain unaffected. This paper presents the conditions for this specific modification, evidence for the nature of the modification, and a study of the effect of this modification upon the functional activity of the tRNA.

Materials

Commercially processed raw wheat germ (*Triticum durum*) was purchased from Shiloh Farms, Sherman, N. Y., and was stored at 4° in plastic bags. Active tRNA, enzymes, and ribosomes were obtained from this material even after storage for over 2 years. tRNA was obtained from the wheat germ by the procedure described by Dudock *et al.* (1969). Yeast tRNA was isolated from baker's yeast (*Saccharomyces cerevisiae*) by the procedure of Holley (1964). Beef liver tRNA was prepared according to Brunngraber (1962). The procedures for preparing purified soluble enzymes from wheat germ, yeast, and beef liver and for preparing wheat germ ribosomes are given by Yoshikami (1970) and are only slightly modified from the procedures used by Leis and Keller (1971).

DEAE-cellulose, type No. 70, capacity 0.9 mequiv/g, was purchased from Carl Schleicher and Schuell Co., Keene, N. H. Benzoylated DEAE-cellulose (BD-cellulose),¹ prepared by the method of Gillam *et al.* (1967), was a gift from Dr. B. S. Dudock. Sephadex G-100 and G-25 were from Pharmacia Fine Chemicals, Inc., Piscataway, N. J. Cellulose thin-layer chromatographic plates (nonfluorescent) were purchased from Analtech, Inc., Wilmington, Del., and Brinkman Instruments, Westbury, N. Y.

RNAse T₁ (EC 2.7.7.26) prepared by Sankyo, Ltd., Tokyo, was purchased from Calbiochem, Los Angeles, Calif. [¹⁴C]-Phenylalanine was obtained from Schwarz BioResearch, Inc., Orangeburg, N. Y. Poly(U) with a sedimentation coefficient of 8.4 S or a number-average molecular weight of about 500,000 (Moore, 1966) was purchased from Miles Chemical Co., Elkhart, Ind.

Methods

Ultraviolet Spectroscopic Analysis. RNA concentrations were measured by absorbance at 260 nm (A_{260}) with a 1-cm light path in neutral solutions containing 10 mM MgCl₂. Amounts of RNA are expressed in A_{260} units: 1 A_{260} unit of RNA in 1 ml of solution gives an A_{260} reading of 1. For pure tRNA^{Phe}, 1 A_{260} unit was taken to be equal to 1.83 nmoles of tRNA^{Phe} (Wimmer *et al.*, 1968).

Fluorescence Assays. All fluorescence measurements were conducted at room temperature in an Aminco-Bowman spectrophotofluorimeter fitted with a xenon arc lamp and an RCA IP-21 photodetector. Standard quartz 1 × 1 cm path-length cuvettes were used. Excitation at 310 nm was used for all mea-

surements in order to minimize inner-filter effects due to the RNA. A minimum number of collimating slits was used, one 1.55-mm entrance slit and one 0.8-mm exit slit, thus sacrificing spectral resolution for sensitivity. To further maximize sensitivity, two stainless steel mirrors were inserted behind the cuvet. The fluorescence intensity reading obtained under these conditions at an emission wavelength of 440 nm is called F_{440} . The F_{440} values reported here were corrected for solvent blanks.

Column Chromatography. The procedures for chromatography of tRNAs on BD-cellulose columns were adaptations of Gillam *et al.* (1967). Oligonucleotides were fractionated on DEAE-cellulose columns in the presence of 7 M urea (Tomlinson and Tener, 1962). The columns (0.4 × 60 cm) were equilibrated with 7 M urea-20 mM Tris·HCl (pH 8 or 8.5). The sample and developing solutions were pumped through the column with a peristaltic pump at a rate of about 0.5 ml/min. The A_{260} of the effluent was monitored on a Gilford recorder Model 2000, Gilford Instrument Inc., Oberlin, Ohio.

Cellulose Thin-Layer Chromatography. Chromatography was carried out at room temperature. Solvents used were as follows (all ratios are expressed in volumes): I, isopropyl alcohol-concentrated NH₄OH-H₂O (7:1:2); II, isobutyric acid-concentrated NH₄OH-H₂O (50:2:28); and III, 1-butanol-concentrated formic acid-H₂O (7:1:2).

Electrophoresis. Flat-bed paper electrophoresis was carried out on Whatman No. 1 paper. Samples were wet spotted along with standards and subjected to a field of about 40 V/cm for about 30 min at 15 to 20°. Final mobilities were calculated relative to the mobility of cytidine at pH 1-3 after corrections for electroendosmosis. Solutions used were: distilled water adjusted to pH 1 with HCl; 10% acetic acid adjusted to pH 2 with formic acid; 0.5% NH₄OH adjusted to pH 3 with formic acid; 0.4% NH₄OH adjusted to pH 4 with formic acid; 0.4% NH₄OH adjusted to pH 5 with acetic acid; 0.5% acetic acid adjusted to pH 6 with pyridine; 0.2% acetic acid adjusted to pH 6.5 with pyridine; 0.05 M NaHCO₃, pH 8.5; 0.05 M Na₂CO₃ adjusted to pH 10 with 0.05 M NaHCO₃; and 0.05 M Na₂CO₃, pH 11.1.

Conversion of tRNA₁^{Phe} into tRNA₂^{Phe} by Treatment with Ammonium Carbonate. Each sample (less than 300 A_{260} units) of tRNA₁^{Phe} was dissolved in 1.5 ml of water. To this was added 1.5 ml of 1 M ammonium carbonate. The final pH was 9. About 20 μ l of CHCl₃ was added to the solution to prevent bacterial growth, and the mixture was incubated at 42° for the desired time. The reaction was terminated by precipitating the tRNA with 6 ml of absolute ethanol. The precipitate was dissolved in 3 ml of water, 0.5 ml of 2 M NaCl was added, and the tRNA was precipitated with 6 ml of absolute ethanol and desiccated.

Excision of the Y Base. Thiebe and Zachau (1968) were the first to demonstrate the acid catalyzed excision of the Y base from yeast tRNA^{Phe}. We used a procedure slightly different from theirs. Dry tRNA^{Phe} or oligonucleotide containing the Y base was taken up either in water adjusted to pH 2.7 with formic acid or HCl, or in 0.1 M ammonium formate (pH 2.7), and incubated in a sealed glass capillary tube at 60° for 30-60 min. The hydrolysate was then spotted directly on a thin-layer plate for chromatography.

Acceptor Assay for tRNA^{Phe}. The assay procedure is given by Yoshikami (1970) and is only slightly modified from the procedure used by Leis and Keller (1971).

Poly(U)-Directed Polyphenylalanine Synthesis. The standard 1-ml reaction mixture consisted of the stated amount of tRNA^{Phe} 10 μ M [¹⁴C]phenylalanine (10 μ Ci/mole), 75

¹ Abbreviations that are not listed in *Biochemistry* 5, 1445 (1966), are: Y_w, Y_y, and Y_b, the fluorescent bases from wheat germ, yeast, and beef liver tRNA^{Phe}s, respectively; BD-cellulose, benzoylated DEAE-cellulose; A_{260} , absorbance at 260 nm with a cell path of 1 cm; F_{440} , fluorescence intensity at 440 nm upon excitation at 310 nm.

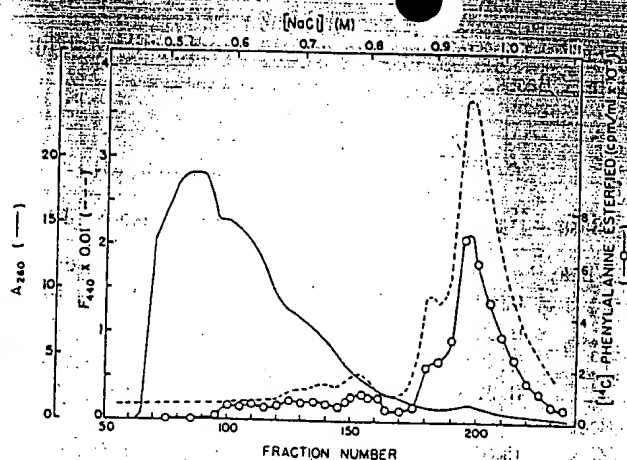


FIGURE 1: BD-cellulose column chromatography of bulk wheat germ tRNA. Bulk wheat germ tRNA (about 300 mg, previously purified by gel filtration on a Sephadex G-100 column) was dissolved in 30 ml of start solution and applied to a BD-cellulose column (1.23 X 90 cm). The column was then eluted with a linear gradient from 0.3 to 1.2 M NaCl containing 10 mM MgCl₂ (1.2-l. total volume). Fractions of 5 ml were collected at a flow rate of 0.5 ml/min.

of poly(U), 50 mM Tris·HCl (pH 7.6), 84 mM KCl, 1 mM EDTA, 10 mM MgCl₂, 0.6 mM GTP, 1 mM ATP, 5 mM phosphoenolpyruvate, 7 A₂₆₀ units of wheat germ ribosomes, and about 1.2 mg of wheat germ soluble fraction enzyme preparation. The mixture was incubated at 37° for the desired time. The reaction was terminated by raising the pH to 11–12 with 0.1 ml of 1 M NaOH, and the mixture was incubated at 37° for 15 min in order to hydrolyze all esterified tRNAs. The solution was neutralized with 0.1 ml of 1 M HCl and then 2.5 ml of 0.4% NaWO₄–8% sodium trichloroacetate (pH 1.7) was added to precipitate the polyphenylalanine. The precipitate was collected and rinsed on a Millipore filter and assayed for ¹⁴C as in the acceptor assay. All data were corrected for blank controls which were treated identically as above except no poly(U) was added.

Results

Fractionation of tRNA^{Phe}s from Wheat Germ. Bulk tRNA from wheat germ was first fractionated on a BD-cellulose column (Gillam *et al.*, 1967) using a linear gradient of NaCl concentration in the presence of 10 mM MgCl₂ (Figure 1). Good resolution was obtained by the use of a slow flow rate and a shallow gradient extending up to 1.2 M NaCl. The column fractions were assayed for fluorescence at 440 nm upon excitation at 310 nm (F₄₄₀) as well as for phenylalanine-acceptor activity. As shown previously (Yoshikami *et al.*, 1968), the F₄₄₀ pattern coincides almost exactly with the elution pattern of the tRNA^{Phe}s. The pattern in Figure 1 shows a number of minor tRNA^{Phe}s in addition to the major species, tRNA₁^{Phe}, which has its peak at tube 197. The small peak in tubes 145–165 is the tRNA₂^{Phe} which was previously detected (Yoshikami *et al.*, 1968) in variable amounts in different bulk wheat germ tRNA preparations.

A hitherto unreported peak, tRNA₃^{Phe}, appears in variable amount on the leading edge of the major peak. tRNA₃^{Phe} was found to be monomeric by gel filtration on Sephadex G-100. The fluorescent Y base obtained by mild acid hydrolysis of this tRNA was chromatographically identical with that from tRNA₁^{Phe}. tRNA₃^{Phe} cochromatographs with tRNA₁^{Phe}

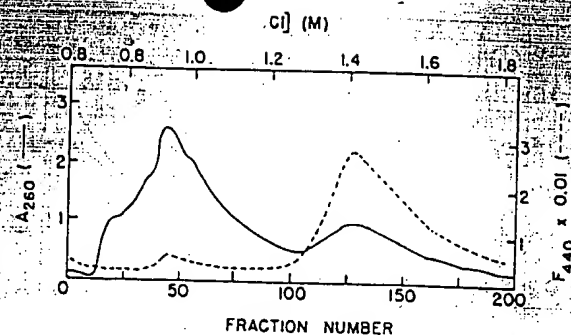


FIGURE 2: Chromatography of partially purified tRNA₁^{Phe} on BD-cellulose at pH 4.5 in the presence of EDTA. Partially purified wheat germ tRNA₁^{Phe} (864 A₂₆₀ units, from chromatography as in Figure 1 of bulk wheat germ soluble RNA not treated by gel filtration) was applied to a BD-cellulose column (1 X 90 cm) and eluted with a linear gradient from 0.8 to 2 M NaCl containing 1 mM EDTA and 10 mM sodium acetate (pH 4.5) (800-ml total volume). Fractions of 4 ml were collected at a flow rate of 1 ml/min. Each tube in the fraction collector contained 0.2 ml of 0.2 M MgCl₂–1 M Tris·HCl (pH 7.5) in order to raise the Mg²⁺ concentration and the pH of the effluent as it emerged from the column.

on BD-cellulose after it has been heated. It therefore appears to be a conformer of tRNA₁^{Phe}.

Purification of tRNA₁^{Phe} from Wheat Germ. The major species, tRNA₁^{Phe}, is obtained from the BD-cellulose column described above at a purity of about 30%. Chromatography of this material on a second BD-cellulose column, this time using a NaCl gradient containing EDTA at pH 4.5, yields tRNA₁^{Phe} that is at least 85% pure as judged by acceptor activity (Figure 2). These two successive column fractionations on BD-cellulose provide a rapid method for obtaining highly purified wheat germ tRNA^{Phe}. This procedure can also be used for obtaining purified rRNA^{Phe} from yeast. A precaution must be observed in using a BD-cellulose column at pH 4.5 with tRNA^{Phe}s which have a Y-type base. As shown by Thiebe and Zachau (1969), the Y base can be excised from the tRNA at low pH. We have noted a very slow excision of Y during chromatography at pH 4.5 at room temperature. To minimize this, a relatively fast flow rate was maintained, and aliquots of Tris buffer at pH 7.5 were placed in the receiving tubes to raise the pH of the effluent (see Figure 2).

Nature of tRNA₂^{Phe} from Wheat Germ. This tRNA has the same fluorescence emission spectrum as tRNA₁^{Phe} (Yoshikami *et al.*, 1968) showing that a Y-type is present. The amount of tRNA₂^{Phe} was variable in different preparations of bulk tRNA suggesting that it was derived from tRNA₁^{Phe} during the isolation of the bulk tRNA. tRNA₂^{Phe} was not simply a conformer of tRNA₁^{Phe} since it was not converted to the latter on heating in solution at 80°. Since tRNA₂^{Phe} had a lower affinity for BD-cellulose than tRNA₁^{Phe}, and since the Y base is responsible for the high affinity of tRNA₁^{Phe} for this resin (*cf.* Thiebe and Zachau, 1969), it was suspected that tRNA₂^{Phe} had been formed by a chemical modification of the Y base.

To test this hypothesis, the Y base from each of the two tRNA^{Phe}s was excised by mild acid treatment (Thiebe and Zachau, 1969) and the chromatographic and electrophoretic mobilities of the two bases were compared. The Y base excised from tRNA₂^{Phe} was found to differ from the base excised from tRNA₁^{Phe}. The former will therefore be designated Y_w² and the latter Y_w¹. In cellulose thin-layer chromatography using organic solvent mixtures, Y_w² has a lower R_f than Y_w¹ (Table I).

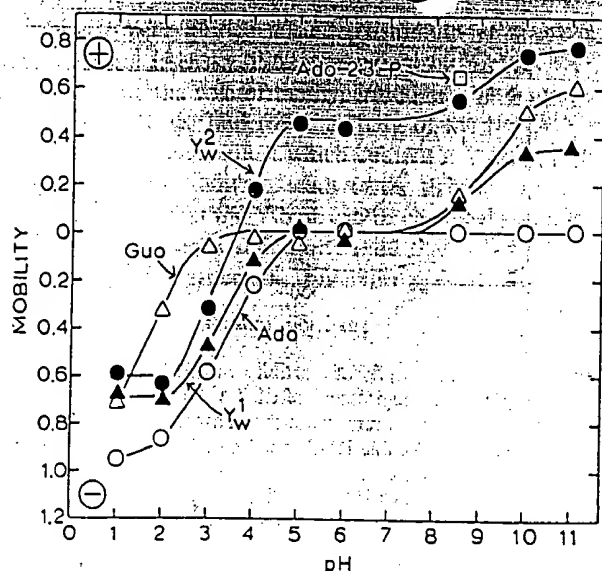


FIGURE 3: Electrophoretic titration of Y_1^1 and Y_2^2 . Y_1^1 and Y_2^2 were obtained by mild acid hydrolysis of wheat germ tRNA^{Phe} and tRNA^{Phe}, respectively, and subsequent thin-layer chromatography in solvent II. Electrophoresis was performed at the various pH's as detailed in Methods.

More information about the difference between Y_1^1 and Y_2^2 was obtained by measuring the electrophoretic mobilities of the two over a wide range of pH values (Figure 3). The electrophoretic titration curves of the two bases are quite different. By comparison to standards, Y_1^1 appears to have a single protonatable group with a pK_a of about 3.3 and a weak acid group with a pK_a near 9. The curve for Y_1^1 is similar to that of guanosine except that the first pK_a of Y_1^1 is higher. A comparison of the curve for Y_2^2 to that of Y_1^1 shows that the latter has an additional titratable group with pK_a = about 4 so that in the neutral pH range (5–7.5) it has a net negative charge. (Y_1^1 has a zero charge in the neutral region.) The results suggest that Y_2^2 has a free carboxyl group on a side chain on the fluorescent chromophore. The net negative charge on Y_2^2 at neutral pH could contribute to the decreased hydrophobicity exhibited by tRNA₂^{Phe} on BD-cellulose columns.

The variable conversion of Y_1^1 into Y_2^2 occurred presumably at some stage during the isolation of the bulk tRNA, possibly during DEAE-cellulose chromatography. The condition of this chromatography may have been inadvertently too alkaline in some cases. RajBhandary *et al.* (1968) and Katz and Dudock (1969) had found that the Y base in oligonucleotides was labile in ammoniacal solvents. An experiment was therefore performed to see if the free base Y_1^1 could be converted to Y_2^2 under mild alkaline conditions. It was found that some conversion did occur when Y_1^1 was incubated in 5 N NH_4OH (pH = about 12) for 12 hr at room temperature. It seems probable that Y_1^1 has an ester group which is hydrolyzed by mild alkaline conditions to yield the free carboxyl group in Y_2^2 .

No large molecular weight change is evident in the conversion of Y_1^1 to Y_2^2 since the electrophoretic mobilities of the two bases are essentially the same at pH 1 where they are both fully protonated (Figure 3). Thus the base-catalyzed conversion could open a lactone ring or release a low molecular weight alcohol.

Conversion of tRNA₁^{Phe} into tRNA₂^{Phe}. If these two RNAs differ from each other only in the Y base, they would be

TABLE I: Chromatographic and Electrophoretic Mobilities of the Y Bases.

Base	R_F in Different Solvents ^a			μ^b at Different pH's			
	I	II	III	2.7	3.5	6.5	10
Wheat							
Y_1^1	0.67	0.90		+0.5	+0.3	0	0
Y_2^2	0.30	0.75	0.31	+0.4	+0.2	-0.5	-0.5
Y_3^3	0.40	0.55		-0.1	-0.1	-0.5	-0.5
Y_4^4	0.22	0.65		+0.8	+0.4	0	0
Beef							
Y_1^1	0.67	0.90			+0.3	0	
Y_2^2	0.30	0.75	0.31	+0.4		-0.5	
Yeast							
Y_1^1	0.84	0.93			+0.3	0	
Y_2^2	0.39	0.77	0.50			-0.5	

^a Cellulose thin-layer chromatography. ^b μ is the electrophoretic mobility taking the mobility of cytidine at pH 1.0, where it has a net charge of +1, as +1.0. ^c Interpolated values from Figure 9.

useful in studying the effect of a specific modification in the hyperon on the function of a tRNA. Conditions were therefore sought to convert Y_1^1 to Y_2^2 *in situ* in the tRNA by base catalysis where no other changes would occur in the tRNA. There are three other modified residues in tRNA^{Phe} which are alkali labile, 7-methylguanosine, 1-methyladenosine, and dihydrouridine. Basic conditions can cause the opening of the imidazole ring of the 7-methylguanosine (Lawley and Brookes, 1963), a rearrangement of 1-methyladenosine to *N*⁶-methyladenosine (Brookes and Lawley, 1960; Macon and Wolfenden, 1968), and a ring opening of dihydrouridine (Green and Cohen, 1957). In each reaction, as is the case for base-catalyzed modification of Y_1^1 , the product has a different charge from the parent compound at neutral pH. These residues appear in different oligonucleotides when the tRNA is digested by RNase T₁. Thus the extent of conversion of each base can be determined by examining the RNase T₁ digest products which have been fractionated on a DEAE-cellulose column in 7 M urea.

It was found that specific modification of the Y base *in situ* could be brought about by treating the tRNA₁^{Phe} with 0.5 M ammonium carbonate (pH 9) at 42° for 12 hr. The conversion to tRNA₂^{Phe} under these conditions was about 50%, as is shown in Figure 4.

In order to establish that the sole difference between these two tRNAs resides in the Y base, an RNase T₁ hydrolysate of each of the tRNAs isolated from the column in Figure 4 was fractionated on a DEAE-cellulose column in the presence of 7 M urea (Figure 5). The elution pattern of the digest of the tRNA₁^{Phe} recovered after the ammonium carbonate treatment (Figure 5A) is virtually identical with that of untreated tRNA₁^{Phe} (Katz and Dudock, 1969). The fluorescence at 440 nm indicates the presence of the Y base in the dodecanucleotide peak 17. The elution pattern of the digest of the tRNA₂^{Phe} (Figure 5B) is identical with that of tRNA₁^{Phe} with the sole exception that the fluorescent dodecanucleotide is shifted to peak 18. Katz and Dudock (1969) had previously detected a small amount of a 12-nucleotide peak in the

A₂₆₀ (—)

FIGURE 4: carboxyl 85% 1 monit 3 ml The c to 2.1 Fract

differ Y ba eluti the n of c treat the c Tl 8.5. ader repe don 196 ader the into mer treat con ader tion / phe wh effi the an dili an ye in to wi reu tk the

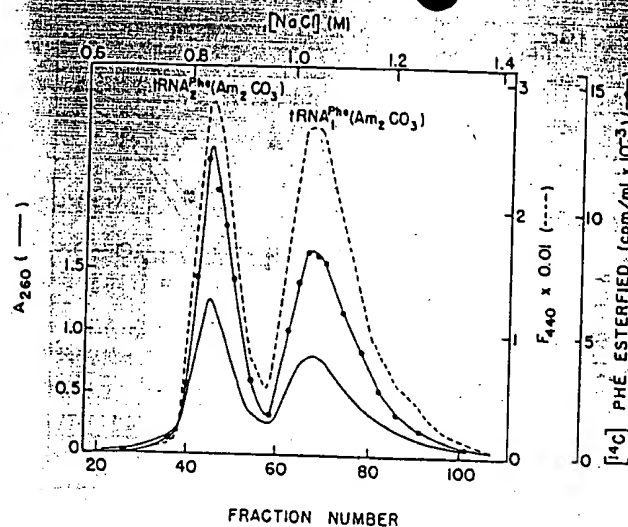


FIGURE 4: BD-cellulose column chromatography of ammonium carbonate treated wheat germ tRNA^{Phe}. tRNA₁^{Phe} (98 A₂₆₀ units, 85% pure) from fractions 120–145 in Figure 2 was treated with ammonium carbonate (pH 9) for 12 hr (see Methods) and applied in 3 ml of start solution to a BD-cellulose column (0.5 × 108 cm). The column was then eluted with a 500-ml linear gradient from 0.5 to 2.0 M NaCl containing 10 mM MgCl₂ (500-ml total volume). Fractions of 3 ml were collected at a flow rate of 0.6 ml/min.

differed from peak 17 only in the fluorescent residue. The Y base excised from peak 18 was found to be Y_w². The later elution of the dodecanucleotide presumably reflects, in part, the negative charge on Y_w². There was no detectable destruction of dihydrouridine or 7-methylguanosine by the alkaline treatment since there was no perturbation of peaks 8 or 10b, the oligonucleotides containing these residues.

The DEAE-cellulose columns in Figure 5 were run at pH 8.5. At this pH there is no charge difference between 1-methyladenosine and N⁶-methyladenosine, so it was necessary to repeat the chromatographic analysis at pH 8. When this was done, peak 15 was resolved into 15a and b (Katz and Dudock, 1969). Peak 15a contains the oligonucleotide with 1-methyladenosine. It could then be estimated that less than 10% of the 1-methyladenosine in the tRNA^{Phe} had been converted into N⁶-methyladenosine by the ammonium carbonate treatment. The analysis at pH 8 was also repeated on tRNA^{Phe} treated with ammonium carbonate for 50 hr. Under this condition a significant amount of modification of 1-methyladenosine and 7-methylguanosine could be detected in addition to some hydrolysis of phosphodiester linkages.

Functional Activity of tRNA₂^{Phe} from Wheat Germ. The phenylalanine-acceptor activity of tRNA₂^{Phe} was demonstrated when it was first detected (Yoshikami *et al.*, 1968). To see what effect the conversion of Y_w¹ into Y_w² has on the interaction of the tRNA with its synthetase, a study was made of the rate of aminoacylation of the two tRNA^{Phe}s (Figure 6). No detectable difference in rate was found. In line with this, Igo-Kemenes and Zachau (1969) have been able to reduce the Y base of yeast tRNA^{Phe} with NaBH₄ with no observable change either in the K_m for the tRNA or the V_{max} of the charging reaction.

It seemed possible that a modification of the base adjacent to the anticodon could influence the interaction of the tRNA with its codon on the ribosome in the course of the transfer reaction. Accordingly a comparison of the transfer function of tRNA^{Phe}s 1 and 2 was made. The two tRNAs were tested for their ability to catalyze the poly(U)-dependent synthesis of polyphenylalanine with ribosomes and crude enzymes from

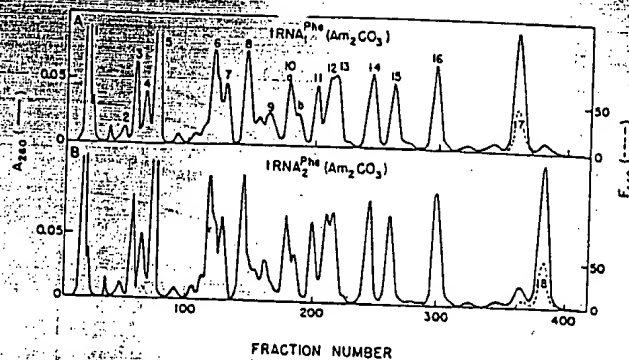


FIGURE 5: Chromatography of RNase T₁ digests of wheat germ tRNA^{Phe} and tRNA₂^{Phe} on DEAE-cellulose. (A) tRNA₁^{Phe} (9 A₂₆₀ units, Am₂CO₃ treated) from the second peak in Figure 4, and (B) tRNA₂^{Phe} (10 A₂₆₀ units, Am₂CO₃ treated) from the first peak in Figure 4 were each digested with 500 units of RNase T₁ in 0.6 ml of 50 mM Tris-HCl (pH 7.7) for 3 hr at 37°. Each digest was then made 7 M in urea, applied to a DEAE-cellulose column (0.4 × 60 cm), and eluted with linear gradient from 0 to 0.3 M NaCl containing 20 mM Tris-HCl (pH 8.5) and 7 M urea (600-ml total volume). Fractions of 1.3 ml were collected at a flow rate of 0.4 ml/min. The peaks are numbered according to Katz and Dudock, 1969.

wheat germ (Figure 7). Under the conditions of the assay, the rate of polymerization was linearly dependent upon the concentration of added tRNA^{Phe}. The optimum pH, temperature, and MgCl₂ concentration for this reaction was found to be the same for both tRNA^{Phe}s. The rate of polymerization catalyzed by tRNA₂^{Phe} was found to be about 70% of that by tRNA₁^{Phe}. This difference in activity was maintained at Mg²⁺ concentrations from 8 to 15 mM, at pH 6–8.5 and at temperatures from 20 to 45°. Thus, the presence of a free-acid group with a negative charge in the hyperon does not abolish the capacity of the tRNA to participate in the transfer reaction, but it does measurably slow the rate at which it functions.

Other Alkaline Degradation Products from Y_w¹. Y_w¹ may be hydrolyzed under alkaline conditions to at least two other forms, Y_w³ and Y_w⁴, in addition to Y_w² (Figure 8). All these forms are readily separated by thin-layer chromatography (Table I). On extended hydrolysis, the Y_w² first formed is converted to Y_w³, and finally the latter is converted into Y_w⁴. All

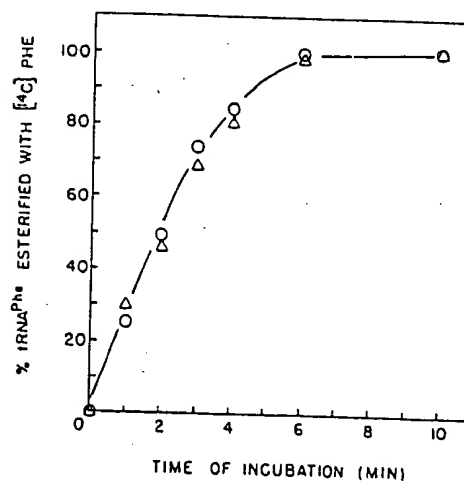


FIGURE 6: Rate of aminoacylation of tRNA₁^{Phe} and tRNA₂^{Phe}. The tRNAs (85% pure, Am₂CO₃ treated from Figure 4) were compared in the acceptor assay (see Methods). Each assay contained 34 μg of wheat germ soluble fraction enzyme preparation in a volume of 0.2 ml. (Δ) tRNA₁^{Phe} (Am₂CO₃), 25 pmole/assay; (O) tRNA₂^{Phe} (Am₂CO₃), 27 pmole/assay.

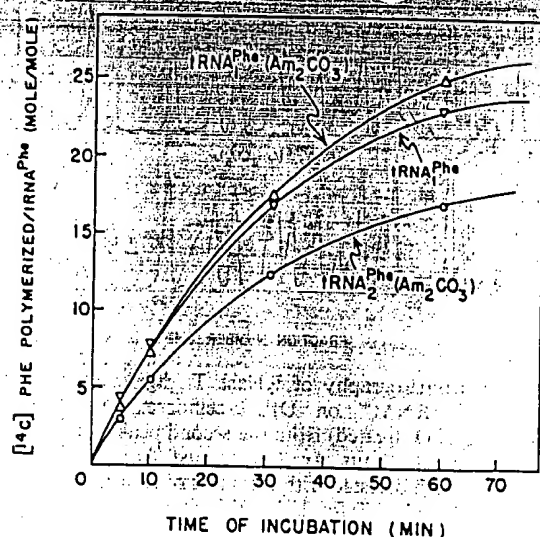


FIGURE 7: Rate of polyphenylalanine synthesis catalyzed by $tRNA^{Phe}$ and $tRNA^{Phe}$. The two treated $tRNA^{Phe}$ (Figure 4) were compared to untreated $tRNA^{Phe}$ (Figure 2) in the wheat germ system for polyphenylalanine synthesis described in Methods. Each tube contained 90 pmoles of $tRNA^{Phe}$ as determined by acceptor assay.

three alkaline degradation products fluoresce and have ultraviolet absorption spectra similar to that of Y_w^1 (cf. Katz and Dudock, 1968). From this it can be inferred that this alkaline hydrolysis has acted on the structure of side chains and not on the fluorescent chromophore of the Y base itself. When the products from an even more extensive alkaline hydrolysis are chromatographed, a number of new ultraviolet-absorbing compounds can be detected. These have different absorption spectra and have lost the characteristic fluorescence of the Y type base.

The electrophoretic mobility of Y_w^3 over a wide pH range is similar to that of Y_w^2 (Figure 9). There is no detectable molecular weight change in the conversion of Y_w^2 into Y_w^3 , and the hydrolysis or rearrangement does not yield any new titratable group. There is, however, a change in chromatographic properties (Table I).

The conversion of Y_w^3 into Y_w^4 results in a marked difference in the electrophoretic titration curve (Figure 9). The alkaline hydrolysis of Y_w^3 to Y_w^4 has released a basic amino group with a pK_a near 9 not present in the other forms of the Y base. This amino group could be blocked by formaldehyde; when 4% formaldehyde was present in the electrophoresis buffer the mobility of Y_w^4 at pH 8.5 was increased to -0.5 . The negative charge indicates that the carboxyl group present in Y_w^3 is still present in Y_w^4 . The mobilities of Y_w^1 , Y_w^2 , and Y_w^3 were not

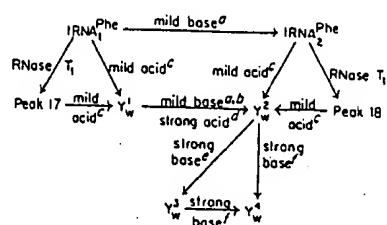


FIGURE 8: Degradation scheme of the Y base of wheat germ $tRNA^{Phe}$. Reaction conditions were as follows: \circ 0.5 M ammonium carbonate (pH 9), 42°, 12 hr; \bullet 5 M NH_4OH , 23°, 12 hr; Δ 0.1 M ammonium formate (pH 3.0) or dilute HCl (pH 3), 60°, 30 min; \square 0.1 M HCl, 100°, 30 min; \times 0.5 M KOH, 60°, 30 min; \circ 0.5 M KOH, 60°, 30 min.

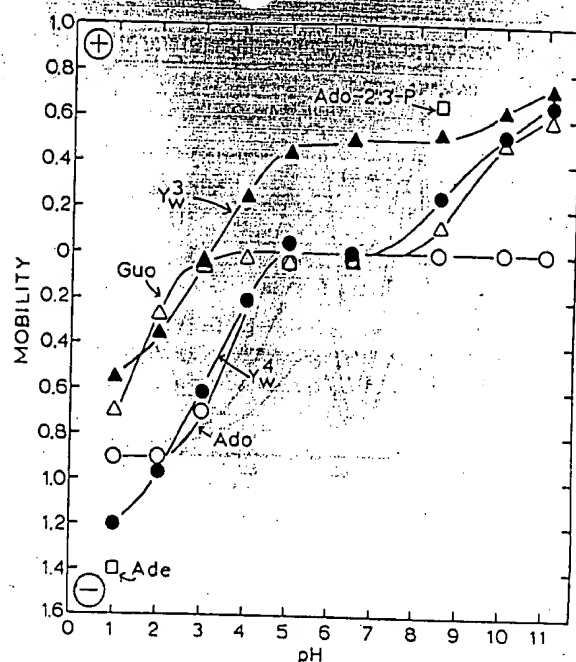


FIGURE 9: Electrophoretic titration of Y^3 and Y^4 . These bases were obtained by KOH hydrolysis of Y^2 (see Figure 8) and subsequent thin-layer chromatography in absolute methanol and then in solvents I and II. Electrophoresis was performed as in Methods.

affected by formaldehyde at pH 8.5. In the absence of formaldehyde, Y_w^4 has a zero mobility at pH's 5-7 where it is a zwitterion. The high mobility of Y_w^4 at pH 1 indicates that it has two positive charges at this pH; one of these charges can be accounted for by the weakly basic group (pK_a = near 3) present also in the other forms of the Y base, the other by the strongly basic group released by hydrolysis. Since the spectrum of the fluorescent chromophore of Y_w^4 is unchanged, the latter group must be on a side chain.

Y Base in $tRNA^{Phe}$ from Beef Liver. When bulk beef liver tRNA is analyzed on a BD-cellulose column as in Figure 1, the pattern of phenylalanine-acceptor activity and F_{440} is almost identical with that for wheat germ (cf. Yoshikami *et al.*, 1968). There is a shoulder of $tRNA^{Phe}$ on the leading edge of the major $tRNA_1^{Phe}$ peak, and this is preceded by a small peak of $tRNA_2^{Phe}$. The Y bases excised from these tRNAs, Y_1^1 and Y_2^1 , appear to be identical with those from the corresponding tRNAs of wheat germ since their respective spectroscopic, chromatographic, and electrophoretic properties are all identical (Table I). When $tRNA_1^{Phe}$ from beef liver (Figure 10A) was incubated in 0.5 M ammonium carbonate (pH 9) for 20 hr at 42° and rechromatographed, about 75% conversion of Y_1^1 into Y_2^1 *in situ* was obtained (Figure 10B). This is about the same amount of conversion that is found with wheat germ $tRNA_1^{Phe}$ under the same conditions.

Y Base in $tRNA^{Phe}$ from Yeast. When bulk yeast tRNA is analyzed on a BD-cellulose column as in Figure 1, the pattern of phenylalanine acceptor activity and F_{440} which is obtained differs from that of wheat germ. In no instance has a peak corresponding to wheat germ $tRNA_3^{Phe}$ been observed. Instead, there is a shoulder of F_{440} and phenylalanine acceptor activity following the major $tRNA_1^{Phe}$ which, like $tRNA_3^{Phe}$ from wheat, may represent a stable conformer of $tRNA_1^{Phe}$. There is a $tRNA_2^{Phe}$ peak in some preparations, but it is usually quite small. When partially purified $tRNA_1^{Phe}$ (Figure 11A) was incubated with 0.5 M ammonium carbonate (pH 9)

FIGURE 9: Electrophoretic titration of Y^3 and Y^4 . These bases were obtained by KOH hydrolysis of Y^2 (see Figure 8) and subsequent thin-layer chromatography in absolute methanol and then in solvents I and II. Electrophoresis was performed as in Methods.

(Figure 9) $tRNA^{Phe}$ (A) $tRNA^{Phe}$ (B) $tRNA^{Phe}$ (C) $tRNA^{Phe}$ (D) $tRNA^{Phe}$ (E) $tRNA^{Phe}$ (F) $tRNA^{Phe}$ (G) $tRNA^{Phe}$ (H) $tRNA^{Phe}$ (I) $tRNA^{Phe}$ (J) $tRNA^{Phe}$ (K) $tRNA^{Phe}$ (L) $tRNA^{Phe}$ (M) $tRNA^{Phe}$ (N) $tRNA^{Phe}$ (O) $tRNA^{Phe}$ (P) $tRNA^{Phe}$ (Q) $tRNA^{Phe}$ (R) $tRNA^{Phe}$ (S) $tRNA^{Phe}$ (T) $tRNA^{Phe}$ (U) $tRNA^{Phe}$ (V) $tRNA^{Phe}$ (W) $tRNA^{Phe}$ (X) $tRNA^{Phe}$ (Y) $tRNA^{Phe}$ (Z) $tRNA^{Phe}$ (AA) $tRNA^{Phe}$ (AB) $tRNA^{Phe}$ (AC) $tRNA^{Phe}$ (AD) $tRNA^{Phe}$ (AE) $tRNA^{Phe}$ (AF) $tRNA^{Phe}$ (AG) $tRNA^{Phe}$ (AH) $tRNA^{Phe}$ (AI) $tRNA^{Phe}$ (AJ) $tRNA^{Phe}$ (AK) $tRNA^{Phe}$ (AL) $tRNA^{Phe}$ (AM) $tRNA^{Phe}$ (AN) $tRNA^{Phe}$ (AO) $tRNA^{Phe}$ (AP) $tRNA^{Phe}$ (AQ) $tRNA^{Phe}$ (AR) $tRNA^{Phe}$ (AS) $tRNA^{Phe}$ (AT) $tRNA^{Phe}$ (AU) $tRNA^{Phe}$ (AV) $tRNA^{Phe}$ (AW) $tRNA^{Phe}$ (AX) $tRNA^{Phe}$ (AY) $tRNA^{Phe}$ (AZ) $tRNA^{Phe}$ (BA) $tRNA^{Phe}$ (BB) $tRNA^{Phe}$ (BC) $tRNA^{Phe}$ (BD) $tRNA^{Phe}$ (BE) $tRNA^{Phe}$ (BF) $tRNA^{Phe}$ (BG) $tRNA^{Phe}$ (BH) $tRNA^{Phe}$ (BI) $tRNA^{Phe}$ (BJ) $tRNA^{Phe}$ (BK) $tRNA^{Phe}$ (BL) $tRNA^{Phe}$ (BM) $tRNA^{Phe}$ (BN) $tRNA^{Phe}$ (BO) $tRNA^{Phe}$ (BP) $tRNA^{Phe}$ (BQ) $tRNA^{Phe}$ (BR) $tRNA^{Phe}$ (BS) $tRNA^{Phe}$ (BT) $tRNA^{Phe}$ (BU) $tRNA^{Phe}$ (BV) $tRNA^{Phe}$ (BW) $tRNA^{Phe}$ (BX) $tRNA^{Phe}$ (BY) $tRNA^{Phe}$ (BZ) $tRNA^{Phe}$ (CA) $tRNA^{Phe}$ (CB) $tRNA^{Phe}$ (CC) $tRNA^{Phe}$ (CD) $tRNA^{Phe}$ (CE) $tRNA^{Phe}$ (CF) $tRNA^{Phe}$ (CG) $tRNA^{Phe}$ (CH) $tRNA^{Phe}$ (CI) $tRNA^{Phe}$ (CJ) $tRNA^{Phe}$ (CK) $tRNA^{Phe}$ (CL) $tRNA^{Phe}$ (CM) $tRNA^{Phe}$ (CN) $tRNA^{Phe}$ (CO) $tRNA^{Phe}$ (CP) $tRNA^{Phe}$ (CQ) $tRNA^{Phe}$ (CR) $tRNA^{Phe}$ (CS) $tRNA^{Phe}$ (CT) $tRNA^{Phe}$ (CU) $tRNA^{Phe}$ (CV) $tRNA^{Phe}$ (CW) $tRNA^{Phe}$ (CX) $tRNA^{Phe}$ (CY) $tRNA^{Phe}$ (CZ) $tRNA^{Phe}$ (DA) $tRNA^{Phe}$ (DB) $tRNA^{Phe}$ (DC) $tRNA^{Phe}$ (DD) $tRNA^{Phe}$ (DE) $tRNA^{Phe}$ (DF) $tRNA^{Phe}$ (DG) $tRNA^{Phe}$ (DH) $tRNA^{Phe}$ (DI) $tRNA^{Phe}$ (DJ) $tRNA^{Phe}$ (DK) $tRNA^{Phe}$ (DL) $tRNA^{Phe}$ (DM) $tRNA^{Phe}$ (DN) $tRNA^{Phe}$ (DO) $tRNA^{Phe}$ (DP) $tRNA^{Phe}$ (DQ) $tRNA^{Phe}$ (DR) $tRNA^{Phe}$ (DS) $tRNA^{Phe}$ (DT) $tRNA^{Phe}$ (DU) $tRNA^{Phe}$ (DV) $tRNA^{Phe}$ (DW) $tRNA^{Phe}$ (DX) $tRNA^{Phe}$ (DY) $tRNA^{Phe}$ (DZ) $tRNA^{Phe}$ (EA) $tRNA^{Phe}$ (EB) $tRNA^{Phe}$ (EC) $tRNA^{Phe}$ (ED) $tRNA^{Phe}$ (EE) $tRNA^{Phe}$ (EF) $tRNA^{Phe}$ (EG) $tRNA^{Phe}$ (EH) $tRNA^{Phe}$ (EI) $tRNA^{Phe}$ (EJ) $tRNA^{Phe}$ (EK) $tRNA^{Phe}$ (EL) $tRNA^{Phe}$ (EM) $tRNA^{Phe}$ (EN) $tRNA^{Phe}$ (EO) $tRNA^{Phe}$ (EP) $tRNA^{Phe}$ (EQ) $tRNA^{Phe}$ (ER) $tRNA^{Phe}$ (ES) $tRNA^{Phe}$ (ET) $tRNA^{Phe}$ (EU) $tRNA^{Phe}$ (EV) $tRNA^{Phe}$ (EW) $tRNA^{Phe}$ (EX) $tRNA^{Phe}$ (EY) $tRNA^{Phe}$ (EZ) $tRNA^{Phe}$ (FA) $tRNA^{Phe}$ (FB) $tRNA^{Phe}$ (FC) $tRNA^{Phe}$ (FD) $tRNA^{Phe}$ (FE) $tRNA^{Phe}$ (FF) $tRNA^{Phe}$ (FG) $tRNA^{Phe}$ (FH) $tRNA^{Phe}$ (FI) $tRNA^{Phe}$ (FJ) $tRNA^{Phe}$ (FK) $tRNA^{Phe}$ (FL) $tRNA^{Phe}$ (FM) $tRNA^{Phe}$ (FN) $tRNA^{Phe}$ (FO) $tRNA^{Phe}$ (FP) $tRNA^{Phe}$ (FQ) $tRNA^{Phe}$ (FR) $tRNA^{Phe}$ (FS) $tRNA^{Phe}$ (FT) $tRNA^{Phe}$ (FU) $tRNA^{Phe}$ (FV) $tRNA^{Phe}$ (FW) $tRNA^{Phe}$ (FX) $tRNA^{Phe}$ (FY) $tRNA^{Phe}$ (FZ) $tRNA^{Phe}$ (GA) $tRNA^{Phe}$ (GB) $tRNA^{Phe}$ (GC) $tRNA^{Phe}$ (GD) $tRNA^{Phe}$ (GE) $tRNA^{Phe}$ (GF) $tRNA^{Phe}$ (GG) $tRNA^{Phe}$ (GH) $tRNA^{Phe}$ (GI) $tRNA^{Phe}$ (GJ) $tRNA^{Phe}$ (GK) $tRNA^{Phe}$ (GL) $tRNA^{Phe}$ (GM) $tRNA^{Phe}$ (GN) $tRNA^{Phe}$ (GO) $tRNA^{Phe}$ (GP) $tRNA^{Phe}$ (GQ) $tRNA^{Phe}$ (GR) $tRNA^{Phe}$ (GS) $tRNA^{Phe}$ (GT) $tRNA^{Phe}$ (GU) $tRNA^{Phe}$ (GV) $tRNA^{Phe}$ (GW) $tRNA^{Phe}$ (GX) $tRNA^{Phe}$ (GY) $tRNA^{Phe}$ (GZ) $tRNA^{Phe}$ (HA) $tRNA^{Phe}$ (HB) $tRNA^{Phe}$ (HC) $tRNA^{Phe}$ (HD) $tRNA^{Phe}$ (HE) $tRNA^{Phe}$ (HF) $tRNA^{Phe}$ (HG) $tRNA^{Phe}$ (HH) $tRNA^{Phe}$ (HI) $tRNA^{Phe}$ (HJ) $tRNA^{Phe}$ (HK) $tRNA^{Phe}$ (HL) $tRNA^{Phe}$ (HM) $tRNA^{Phe}$ (HN) $tRNA^{Phe}$ (HO) $tRNA^{Phe}$ (HP) $tRNA^{Phe}$ (HQ) $tRNA^{Phe}$ (HR) $tRNA^{Phe}$ (HS) $tRNA^{Phe}$ (HT) $tRNA^{Phe}$ (HU) $tRNA^{Phe}$ (HV) $tRNA^{Phe}$ (HW) $tRNA^{Phe}$ (HX) $tRNA^{Phe}$ (HY) $tRNA^{Phe}$ (HZ) $tRNA^{Phe}$ (IA) $tRNA^{Phe}$ (IB) $tRNA^{Phe}$ (IC) $tRNA^{Phe}$ (ID) $tRNA^{Phe}$ (IE) $tRNA^{Phe}$ (IF) $tRNA^{Phe}$ (IG) $tRNA^{Phe}$ (IH) $tRNA^{Phe}$ (II) $tRNA^{Phe}$ (IJ) $tRNA^{Phe}$ (IK) $tRNA^{Phe}$ (IL) $tRNA^{Phe}$ (IM) $tRNA^{Phe}$ (IN) $tRNA^{Phe}$ (IO) $tRNA^{Phe}$ (IP) $tRNA^{Phe}$ (IQ) $tRNA^{Phe}$ (IR) $tRNA^{Phe}$ (IS) $tRNA^{Phe}$ (IT) $tRNA^{Phe}$ (IU) $tRNA^{Phe}$ (IV) $tRNA^{Phe}$ (IW) $tRNA^{Phe}$ (IX) $tRNA^{Phe}$ (IY) $tRNA^{Phe}$ (IZ) $tRNA^{Phe}$ (JA) $tRNA^{Phe}$ (JB) $tRNA^{Phe}$ (JC) $tRNA^{Phe}$ (JD) $tRNA^{Phe}$ (JE) $tRNA^{Phe}$ (JF) $tRNA^{Phe}$ (JG) $tRNA^{Phe}$ (JH) $tRNA^{Phe}$ (JI) $tRNA^{Phe}$ (JJ) $tRNA^{Phe}$ (JK) $tRNA^{Phe}$ (JL) $tRNA^{Phe}$ (JM) $tRNA^{Phe}$ (JN) $tRNA^{Phe}$ (JO) $tRNA^{Phe}$ (JP) $tRNA^{Phe}$ (JQ) $tRNA^{Phe}$ (JR) $tRNA^{Phe}$ (JS) $tRNA^{Phe}$ (JT) $tRNA^{Phe}$ (JU) $tRNA^{Phe}$ (JV) $tRNA^{Phe}$ (JW) $tRNA^{Phe}$ (JX) $tRNA^{Phe}$ (JY) $tRNA^{Phe}$ (JZ) $tRNA^{Phe}$ (KA) $tRNA^{Phe}$ (KB) $tRNA^{Phe}$ (KC) $tRNA^{Phe}$ (KD) $tRNA^{Phe}$ (KE) $tRNA^{Phe}$ (KF) $tRNA^{Phe}$ (KG) $tRNA^{Phe}$ (KH) $tRNA^{Phe}$ (KI) $tRNA^{Phe}$ (KJ) $tRNA^{Phe}$ (KL) $tRNA^{Phe}$ (KM) $tRNA^{Phe}$ (KN) $tRNA^{Phe}$ (KO) $tRNA^{Phe}$ (KP) $tRNA^{Phe}$ (KQ) $tRNA^{Phe}$ (KR) $tRNA^{Phe}$ (KS) $tRNA^{Phe}$ (KT) $tRNA^{Phe}$ (KU) $tRNA^{Phe}$ (KV) $tRNA^{Phe}$ (KW) $tRNA^{Phe}$ (KX) $tRNA^{Phe}$ (KY) $tRNA^{Phe}$ (KZ) $tRNA^{Phe}$ (LA) $tRNA^{Phe}$ (LB) $tRNA^{Phe}$ (LC) $tRNA^{Phe}$ (LD) $tRNA^{Phe}$ (LE) $tRNA^{Phe}$ (LF) $tRNA^{Phe}$ (LG) $tRNA^{Phe}$ (LH) $tRNA^{Phe}$ (LI) $tRNA^{Phe}$ (LJ) $tRNA^{Phe}$ (LK) $tRNA^{Phe}$ (LL) $tRNA^{Phe}$ (LM) $tRNA^{Phe}$ (LN) $tRNA^{Phe}$ (LO) $tRNA^{Phe}$ (LP) $tRNA^{Phe}$ (LQ) $tRNA^{Phe}$ (LR) $tRNA^{Phe}$ (LS) $tRNA^{Phe}$ (LT) $tRNA^{Phe}$ (LU) $tRNA^{Phe}$ (LV) $tRNA^{Phe}$ (LW) $tRNA^{Phe}$ (LX) $tRNA^{Phe}$ (LY) $tRNA^{Phe}$ (LZ) $tRNA^{Phe}$ (MA) $tRNA^{Phe}$ (MB) $tRNA^{Phe}$ (MC) $tRNA^{Phe}$ (MD) $tRNA^{Phe}$ (ME) $tRNA^{Phe}$ (MF) $tRNA^{Phe}$ (MG) $tRNA^{Phe}$ (MH) $tRNA^{Phe}$ (MI) $tRNA^{Phe}$ (MJ) $tRNA^{Phe}$ (MK) $tRNA^{Phe}$ (ML) $tRNA^{Phe}$ (MM) $tRNA^{Phe}$ (MN) $tRNA^{Phe}$ (MO) $tRNA^{Phe}$ (MP) $tRNA^{Phe}$ (MQ) $tRNA^{Phe}$ (MR) $tRNA^{Phe}$ (MS) $tRNA^{Phe}$ (MT) $tRNA^{Phe}$ (MU) $tRNA^{Phe}$ (MV) $tRNA^{Phe}$ (MW) $tRNA^{Phe}$ (MX) $tRNA^{Phe}$ (MY) $tRNA^{Phe}$ (MZ) $tRNA^{Phe}$ (NA) $tRNA^{Phe}$ (NB) $tRNA^{Phe}$ (NC) $tRNA^{Phe}$ (ND) $tRNA^{Phe}$ (NE) $tRNA^{Phe}$ (NF) $tRNA^{Phe}$ (NG) $tRNA^{Phe}$ (NH) $tRNA^{Phe}$ (NI) $tRNA^{Phe}$ (NJ) $tRNA^{Phe}$ (NK) $tRNA^{Phe}$ (NL) $tRNA^{Phe}$ (NM) $tRNA^{Phe}$ (NN) $tRNA^{Phe}$ (NO) $tRNA^{Phe}$ (NP) $tRNA^{Phe}$ (NQ) $tRNA^{Phe}$ (NR) $tRNA^{Phe}$ (NS) $tRNA^{Phe}$ (NT) $tRNA^{Phe}$ (NU) $tRNA^{Phe}$ (NV) $tRNA^{Phe}$ (NW) $tRNA^{Phe}$ (NX) $tRNA^{Phe}$ (NY) $tRNA^{Phe}$ (NZ) $tRNA^{Phe}$ (OA) $tRNA^{Phe}$ (OB) $tRNA^{Phe}$ (OC) $tRNA^{Phe}$ (OD) $tRNA^{Phe}$ (OE) $tRNA^{Phe}$ (OF) $tRNA^{Phe}$ (OG) $tRNA^{Phe}$ (OH) $tRNA^{Phe}$ (OI) $tRNA^{Phe}$ (OJ) $tRNA^{Phe}$ (OK) $tRNA^{Phe}$ (OL) $tRNA^{Phe}$ (OM) $tRNA^{Phe}$ (ON) $tRNA^{Phe}$ (OO) $tRNA^{Phe}$ (OP) $tRNA^{Phe}$ (OQ) $tRNA^{Phe}$ (OR) $tRNA^{Phe}$ (OS) $tRNA^{Phe}$ (OT) $tRNA^{Phe}$ (OU) $tRNA^{Phe}$ (OV) $tRNA^{Phe}$ (OW) $tRNA^{Phe}$ (OX) $tRNA^{Phe}$ (OY) $tRNA^{Phe}$ (OZ) $tRNA^{Phe}$ (PA) $tRNA^{Phe}$ (PB) $tRNA^{Phe}$ (PC) $tRNA^{Phe}$ (PD) $tRNA^{Phe}$ (PE) $tRNA^{Phe}$ (PF) $tRNA^{Phe}$ (PG) $tRNA^{Phe}$ (PH) $tRNA^{Phe}$ (PI) $tRNA^{Phe}$ (PJ) $tRNA^{Phe}$ (PK) $tRNA^{Phe}$ (PL) $tRNA^{Phe}$ (PM) $tRNA^{Phe}$ (PN) $tRNA^{Phe}$ (PO) $tRNA^{Phe}$ (PP) $tRNA^{Phe}$ (PQ) $tRNA^{Phe}$ (PR) $tRNA^{Phe}$ (PS) $tRNA^{Phe}$ (PT) $tRNA^{Phe}$ (PU) $tRNA^{Phe}$ (PV) $tRNA^{Phe}$ (PW) $tRNA^{Phe}$ (PX) $tRNA^{Phe}$ (PY) $tRNA^{Phe}$ (PZ) $tRNA^{Phe}$ (QA) $tRNA^{Phe}$ (QB) $tRNA^{Phe}$ (QC) $tRNA^{Phe}$ (QD) $tRNA^{Phe}$ (QE) $tRNA^{Phe}$ (QF) $tRNA^{Phe}$ (QG) $tRNA^{Phe}$ (QH) $tRNA^{Phe}$ (QI) $tRNA^{Phe}$ (QJ) $tRNA^{Phe}$ (QK) $tRNA^{Phe}$ (QL) $tRNA^{Phe}$ (QM) $tRNA^{Phe}$ (QN) $tRNA^{Phe}$ (QO) $tRNA^{Phe}$ (QP) $tRNA^{Phe}$ (QQ) $tRNA^{Phe}$ (QR) $tRNA^{Phe}$ (QS) $tRNA^{Phe}$ (QT) $tRNA^{Phe}$ (QU) $tRNA^{Phe}$ (QV) $tRNA^{Phe}$ (QW) $tRNA^{Phe}$ (QX) $tRNA^{Phe}$ (QY) $tRNA^{Phe}$ (QZ) $tRNA^{Phe}$ (RA) $tRNA^{Phe}$ (RB) $tRNA^{Phe}$ (RC) $tRNA^{Phe}$ (RD) $tRNA^{Phe}$ (RE) $tRNA^{Phe}$ (RF) $tRNA^{Phe}$ (RG) $tRNA^{Phe}$ (RH) $tRNA^{Phe}$ (RI) $tRNA^{Phe}$ (RJ) $tRNA^{Phe}$ (RK) $tRNA^{Phe}$ (RL) $tRNA^{Phe}$ (RM) $tRNA^{Phe}$ (RN) $tRNA^{Phe}$ (RO) $tRNA^{Phe}$ (RP) $tRNA^{Phe}$ (RQ) $tRNA^{Phe}$ (RR) $tRNA^{Phe}$ (RS) $tRNA^{Phe}$ (RT) $tRNA^{Phe}$ (RU) $tRNA^{Phe}$ (RV) $tRNA^{Phe}$ (RW) $tRNA^{Phe}$ (RX) $tRNA^{Phe}$ (RY) $tRNA^{Phe}$ (RZ) $tRNA^{Phe}$ (SA) $tRNA^{Phe}$ (SB) $tRNA^{Phe}$ (SC) $tRNA^{Phe}$ (SD) $tRNA^{Phe}$ (SE) $tRNA^{Phe}$ (SF) $tRNA^{Phe}$ (SG) $tRNA^{Phe}$ (SH) $tRNA^{Phe}$ (SI) $tRNA^{Phe}$ (SJ) $tRNA^{Phe}$ (SK) $tRNA^{Phe}$ (SL) $tRNA^{Phe}$ (SM) $tRNA^{Phe}$ (SN) $tRNA^{Phe}$ (SO) $tRNA^{Phe}$ (SP) $tRNA^{Phe}$ (SQ) $tRNA^{Phe}$ (SR) $tRNA^{Phe}$ (SS) $tRNA^{Phe}$ (ST) $tRNA^{Phe}$ (SU) $tRNA^{Phe}$ (SV) $tRNA^{Phe}$ (SW) $tRNA^{Phe}$ (SX) $tRNA^{Phe}$ (SY) $tRNA^{Phe}$ (SZ) $tRNA^{Phe}$ (TA) $tRNA^{Phe}$ (TB) $tRNA^{Phe}$ (TC) $tRNA^{Phe}$ (TD) $tRNA^{Phe}$ (TE) $tRNA^{Phe}$ (TF) $tRNA^{Phe}$ (TG) $tRNA^{Phe}$ (TH) $tRNA^{Phe}$ (TI) $tRNA^{Phe}$ (TJ) $tRNA^{Phe}$ (TK) $tRNA^{Phe}$ (TL) $tRNA^{Phe}$ (TM) $tRNA^{Phe}$ (TN) $tRNA^{Phe}$ (TO) $tRNA^{Phe}$ (TP) $tRNA^{Phe}$ (TQ) $tRNA^{Phe}$ (TR) $tRNA^{Phe}$ (TS) $tRNA^{Phe}$ (TT) $tRNA^{Phe}$ (TU) $tRNA^{Phe}$ (TV) $tRNA^{Phe}$ (TW) $tRNA^{Phe}$ (TX) $tRNA^{Phe}$ (TY) $tRNA^{Phe}$ (TZ) $tRNA^{Phe}$ (UA) $tRNA^{Phe}$ (UB) $tRNA^{Phe}$ (UC) $tRNA^{Phe}$ (UD) $tRNA^{Phe}$ (UE) $tRNA^{Phe}$ (UF) $tRNA^{Phe}$ (UG) $tRNA^{Phe}$ (UH) $tRNA^{Phe}$ (UI) $tRNA^{Phe}$ (UJ) $tRNA^{Phe}$ (UK) $tRNA^{Phe}$ (UL) $tRNA^{Phe}$ (UM) $tRNA^{Phe}$ (UN) $tRNA^{Phe}$ (UO) $tRNA^{Phe}$ (UP) $tRNA^{Phe}$ (UQ) $tRNA^{Phe}$ (UR) $tRNA^{Phe}$ (US) $tRNA^{Phe}$ (UT) $tRNA^{Phe}$ (UU) $tRNA^{Phe}$ (UV) $tRNA^{Phe}$ (UW) $tRNA^{Phe}$ (UX) $tRNA^{Phe}$ (UY) $tRNA^{Phe}$ (UZ) $tRNA^{Phe}$ (VA) $tRNA^{Phe}$ (VB) $tRNA^{Phe}$ (VC) $tRNA^{Phe}$ (VD) $tRNA^{Phe}$ (VE) $tRNA^{Phe}$ (VF) $tRNA^{Phe}$ (VG) $tRNA^{Phe}$ (VH) $tRNA^{Phe}$ (VI) $tRNA^{Phe}$ (VJ) $tRNA^{Phe}$ (VK) $tRNA^{Phe}$ (VL) $tRNA^{Phe}$ (VM) $tRNA^{Phe}$ (VN) $tRNA^{Phe}$ (VO) $tRNA^{Phe}$ (VP) $tRNA^{Phe}$ (VQ) $tRNA^{Phe}$ (VR) $tRNA^{Phe}$ (VS) $tRNA^{Phe}$ (VT) $tRNA^{Phe}$ (VU) $tRNA^{Phe}$ (VV) $tRNA^{Phe}$ (VW) $tRNA^{Phe}$ (VX) $tRNA^{Phe}$ (VY) $tRNA^{Phe}$ (VZ) $tRNA^{Phe}$ (WA) $tRNA^{Phe}$ (WB) $tRNA^{Phe}$ (WC) $tRNA^{Phe}$ (WD) $tRNA^{Phe}$ (WE) $tRNA^{Phe}$ (WF) $tRNA^{Phe}$ (WG) $tRNA^{Phe}$ (WH) $tRNA^{Phe}$ (WI) $tRNA^{Phe}$ (WJ) $tRNA^{Phe}$ (WK) $tRNA^{Phe}$ (WL) $tRNA^{Phe}$ (WM) $tRNA^{Phe}$ (WN) $tRNA^{Phe}$ (WO) $tRNA^{Phe}$ (WP) $tRNA^{Phe}$ (WQ) $tRNA^{Phe}$ (WR) $tRNA^{Phe}$ (WS) $tRNA^{Phe}$ (WT) $tRNA^{Phe}$ (WU) $tRNA^{Phe}$ (WV) $tRNA^{Phe}$ (WW) $tRNA^{Phe}$ (WX) $tRNA^{Phe}$ (WY) $tRNA^{Phe}$ (WZ) $tRNA^{Phe}$ (XA) $tRNA^{Phe}$ (XB) $tRNA^{Phe}$ (XC) $tRNA^{Phe}$ (XD) $tRNA^{Phe}$ (XE) $tRNA^{Phe}$ (XF) $tRNA^{Phe}$ (XG) $tRNA^{Phe}$ (XH) $tRNA^{Phe}$ (XI) $tRNA^{Phe}$ (XJ) $tRNA^{Phe}$ (XK) $tRNA^{Phe}$ (XL) $tRNA^{Phe}$ (XM) $tRNA^{Phe}$ (XN) $tRNA^{Phe}$ (XO) $tRNA^{Phe}$ (XP) $tRNA^{Phe}$ (XQ) $tRNA^{Phe}$ (XR) $tRNA^{Phe}$ (XS) $tRNA^{Phe}$ (XT) $tRNA^{Phe}$ (XU) $tRNA^{Phe}$ (XV) $tRNA^{Phe}$ (XW) $tRNA^{Phe}$ (XX) $tRNA^{Phe}$ (XY) $tRNA^{Phe}$ (XZ) $tRNA^{Phe}$ (YA) $tRNA^{Phe}$ (YB) $tRNA^{Phe}$ (YC) $tRNA^{Phe}$ (YD) $tRNA^{Phe}$ (YE) $tRNA^{Phe}$ (YF) $tRNA^{Phe}$ (YG) $tRNA^{Phe}$ (YH) $tRNA^{Phe}$ (YI) $tRNA^{Phe}$ (YJ) $tRNA^{Phe}$ (YK) $tRNA^{Phe}$ (YL) $tRNA^{Phe}$ (YM) $tRNA^{Phe}$ (YN) $tRNA^{Phe}$ (YO) $tRNA^{Phe}$ (YP) $tRNA^{Phe}$ (YQ) $tRNA^{Phe}$ (YR) $tRNA^{Phe}$ (YS) $tRNA^{Phe}$ (YT) $tRNA^{Phe}$ (YU) $tRNA^{Phe}$ (YV) $tRNA^{Phe}$ (YW) $tRNA^{Phe}$ (YX) $tRNA^{Phe}$ (YY) $tRNA^{Phe}$ (YZ) $tRNA^{Phe}$ (ZA) $tRNA^{Phe}$ (ZB) $tRNA^{Phe}$ (ZC) $tRNA^{Phe}$ (ZD) $tRNA^{Phe}$ (ZE) $tRNA^{Phe}$ (ZF) $tRNA^{Phe}$ (ZG) $tRNA^{Phe}$ (ZH) $tRNA^{Phe}$ (ZI) $tRNA^{Phe}$ (ZJ) $tRNA^{Phe}$ (ZK) $tRNA^{Phe}$ (ZL) $tRNA^{Phe}$ (ZM) $tRNA^{Phe}$ (ZN) $tRNA^{Phe}$ (ZO) $tRNA^{Phe}$ (ZP) $tRNA^{Phe}$ (ZQ) $tRNA^{Phe}$ (ZR) $tRNA^{Phe}$ (ZS) $tRNA^{Phe}$ (ZT) $tRNA^{Phe}$ (ZU) $tRNA^{Phe}$ (ZV) $tRNA^{Phe}$ (ZW) $tRNA^{Phe}$ (ZX) $tRNA^{Phe}$ (ZY) $tRNA^{Phe}$ (ZZ) $tRNA^{Phe}$ (AA) $tRNA^{Phe}$ (AB) $tRNA^{Phe}$ (AC) $tRNA^{Phe}$ (AD) $tRNA^{Phe}$ (AE) $tRNA^{Phe}$ (AF) $tRNA^{Phe}$ (AG) $tRNA^{Phe}$ (AH) $tRNA^{Phe}$ (AI) $tRNA^{Phe}$ (AJ) $tRNA^{Phe}$ (AK) $tRNA^{Phe}$ (AL) $tRNA^{Phe}$ (AM) $tRNA^{Phe}$ (AN) $tRNA^{Phe$

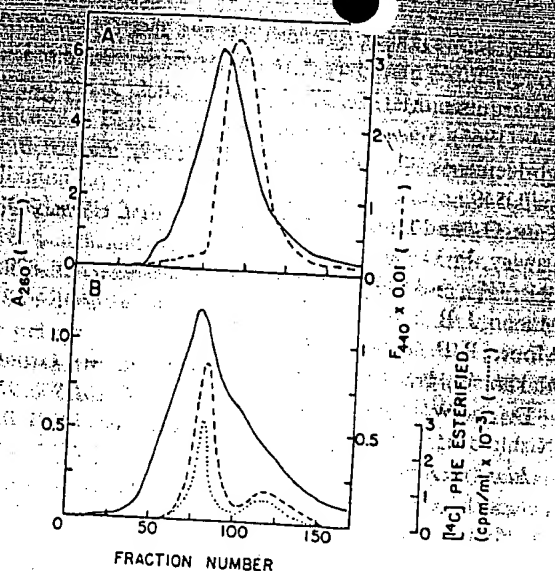


FIGURE 10: BD-cellulose column chromatography of beef liver tRNA^{Phe} before and after treatment with ammonium carbonate. (A) Partially purified beef liver tRNA^{Phe} (950 A_{260} units, from a BD-cellulose column as in Figure 1) was dissolved in 10 ml of start solution, applied to a BD-cellulose column (1 \times 45 cm), and eluted with a linear gradient from 0.5 to 2.4 M NaCl containing 10 mM MgCl₂ (500-ml total volume). Fractions of 3 ml were collected at a flow rate of 1 ml/min. (B) Half of the RNA recovered from fractions 90 to 116 in part A was treated with ammonium carbonate for 20 hr (see Methods) and chromatographed as in part A.

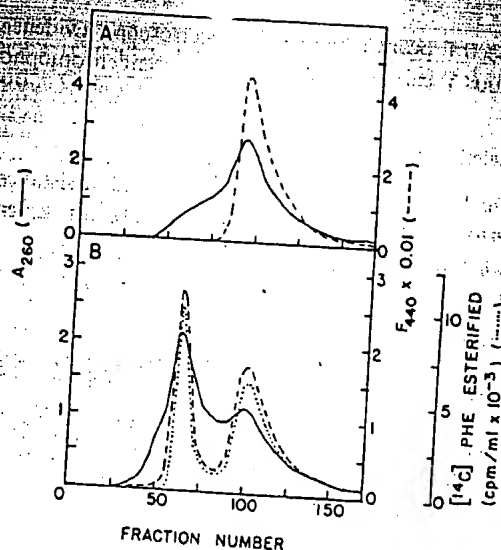


FIGURE 11: BD-cellulose column chromatography of yeast tRNA^{Phe} before and after ammonium carbonate treatment. (A) Partially purified yeast tRNA^{Phe} (330 A_{260} units, from a BD-cellulose column as in Figure 1) was chromatographed on BD-cellulose as in Figure 10A. (B) Another portion of the same tRNA (270 A_{260} units) was incubated with ammonium carbonate for 20 hr (see Methods) and chromatographed in the same way.

(Figure 11B). Acid excision of the Y bases from tRNA₁^{Phe} and tRNA₂^{Phe} yielded Y₁¹ and Y₂², respectively (Table I). This conversion was slower than in the case of beef and wheat germ. Furthermore, the base Y₁¹ is itself more resistant to base-catalyzed conversion into a Y₂² base than is Y_w¹ or Y_b¹. However, for all Y bases, the conversion to a Y₂² base exposes a free acidic group (Table I). It appears then that the susceptible linkage in Y₁¹ is more stable to alkali than that in Y_w¹ or Y_b¹. Both Y₁¹ and Y₂² are chromatographically distinguishable from their counterparts in wheat and beef. This indicates that some substituent on the chromophores of these bases differ though the fluorescent chromophores themselves may be identical as judged by their similar spectroscopic characteristics (RajBhandary *et al.*, 1968; Katz and Dudock, 1969; Yoshikami *et al.*, 1968).

Discussion

To understand the role of the hypermodified base adjacent to the anticodon of different tRNAs, we chose to study the tRNA^{Phe}s which have the fluorescent Y-type base in this locus. We were able to isolate three products of the wheat germ Y base which retain the fluorescent chromophore and represent progressive stages of alkaline hydrolysis. The first stage of hydrolysis, the conversion of Y_w¹ into Y_w², can be effected *in situ* under a very mild alkaline condition which has no effect on any other part of the tRNA^{Phe}. This was used to generate a tRNA^{Phe} specifically modified in the anticodon-adjacent base, namely tRNA₂^{Phe}. Our analysis indicates that the modification produces a net negative charge on the Y base.

The acceptor activity of wheat germ tRNA₂^{Phe} was comparable to that of the naturally occurring tRNA₁^{Phe}. On the other hand, tRNA₂^{Phe} was less efficient than tRNA₁^{Phe} in supporting the polymerization of phenylalanine coded for by poly(U). tRNA₂^{Phe} catalyzed this reaction at 70% the rate of tRNA₁^{Phe}.

Thus, although the modification of the tRNA^{Phe} does not affect the acceptor activity of the tRNA, it does significantly alter its efficiency in the transfer reaction when tested *in vitro* with poly(U). Further analysis would be required to determine the efficiency of the transfer reaction with a natural mRNA and whether the modification affects the fidelity of translation.

These results are congruent with those obtained by a number of other workers who have demonstrated that the structural integrity of the anticodon-adjacent base is necessary for optimum functioning of the tRNA in the transfer reaction, but that modification of this base does not critically affect acceptor activity (Fittler and Hall, 1966; Thiebe and Zachau, 1968; Geffer and Russell, 1969; Ghosh and Ghosh, 1970; Furuichi *et al.*, 1970).

Nakanishi *et al.* (1970) have recently proposed a structure for the Y base from yeast tRNA^{Phe} (Y₁¹). In this structure the fluorescent chromophore bears an α -amino acid side chain. The amino group is blocked with a carbomethoxy moiety, and the carboxyl group is present as a methyl ester. The carboxyl group released in the conversion of Y₁¹ into Y₂² is undoubtedly the latter carboxyl group. The release of a carboxyl group in forming Y_w² and of a free amino group in forming Y_w⁴ indicates that the wheat germ Y base has a blocked α -amino acid side chain similar to that of yeast Y.

There is a difference, however, both in chromatographic behavior and rate of hydrolysis between the yeast Y and wheat germ Y (and the apparently identical beef liver Y). Furthermore, there appears to be some structural feature in wheat germ Y not present in the proposed structure of Y₁¹ which is responsible for the formation of the additional intermediate, Y_w³. That the difference occurs in the distal portion of the side chain is indicated by a low-resolution mass spectral analysis of Y_w¹ (Yoshikami, 1970). Prominent peaks at m/e 216 and 230 were observed just as in the analysis of Y₁¹ by Nakanishi *et al.* (1970). The latter authors showed that these two peaks represented the fluorescent chromophore with one and two carbons of the side chain, respectively.

mass spectral peaks supports the spectroscopic evidence that the various Y bases have identical fluorescent chromophores.

Acknowledgments

We thank Mrs. Clara T. Kahn for technical assistance, and Jr. Bernard Dudock and Mr. Gary Katz for generously supplying materials used in this study.

References

- Barrell, B. G., and Sanger, F. (1969), *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 3, 275.
- Biemann, K., Tsunakawa, S., Sonnenbichler, J., Feldmann, H., Dütting, D., and Zachau, H. G. (1966), *Angew. Chem.* 78, 600.
- Brookes, P., and Lawley, P. D. (1960), *J. Chem. Soc.*, 539.
- Brunngraber, E. B. (1962), *Biochem. Biophys. Res. Commun.* 8, 1.
- Burrows, W. G., Armstrong, D. J., Skoog, F., Hecht, S. M., Boyle, J. T. A., Leonard, N. J., and Occolowitz, J. (1968), *Science* 156, 691.
- Dudock, B. S., Katz, G., Taylor, E. K., and Holley, R. W. (1969), *Proc. Nat. Acad. Sci. U. S.* 62, 941.
- Fink, L. M., Goto, T., Frankel, F., and Weinstein, I. B. (1968), *Biochem. Biophys. Res. Commun.* 32, 963.
- Fittler, F., and Hall, R. H. (1966), *Biochem. Biophys. Res. Commun.* 25, 441.
- Furuichi, Y., Wataya, Y., Hayatsu, H., and Ukita, T. (1970), *Biochem. Biophys. Res. Commun.* 41, 1185.
- Gefter, M. L., and Russell, R. L. (1969), *J. Mol. Biol.* 39, 145.
- Ghosh, K., and Ghosh, H. P. (1970), *Biochem. Biophys. Res. Commun.* 40, 135.
- Ilam, I., Millward, S., Blew, D., von Tigerstrom, M., Wimmer, E., and Tener, G. M. (1967), *Biochemistry* 6, 3043.
- Green, M., and Cohen, S. S. (1957), *J. Biol. Chem.* 225, 397.

- Holley, R. W. (1964), *Biochem. Biophys. Res. Commun.* 10, 186.
- Holley, R. W., Appgar, J., Everett, G. A., Madison, J. T., Marquisee, M., Merrill, S. H., Penswick, J. R., and Zamir, A. (1965), *Science* 147, 1462.
- Igo-Kemenes, T., and Zachau, H. (1969), *Eur. J. Biochem.* 10, 549.
- Katz, G., and Dudock, B. S. (1969), *J. Biol. Chem.* 244, 3062.
- Lawley, P. D., and Brookes, P. (1963), *Biochem. J.* 89, 127.
- Leis, J. P., and Keller, E. B. (1971), *Biochemistry* 10, 889.
- Macon, J. B., and Wolfenden, R. (1968), *Biochemistry* 7, 3453.
- Moore, P. B. (1966), *J. Mol. Biol.* 18, 8.
- Nakanishi, K., Furutachi, N., Funamizu, M., Grunberger, D., and Weinstein, I. B. (1970), *J. Amer. Chem. Soc.* 92, 7617.
- Nishimura, S., Yamada, Y., and Ishikura, H. (1969), *Biochim. Biophys. Acta* 179, 517.
- Peterkofsky, A., and Jesensky, C. (1969), *Biochemistry* 8, 3798.
- RajBhandary, U. L., Faulkner, R. D., and Stuart, A. (1968), *J. Biol. Chem.* 243, 575.
- RajBhandary, U. L., Stuart, A., Faulkner, R. D., Chang, S. H., and Khorana, H. G. (1967), *Proc. Nat. Acad. Sci. U. S.* 57, 751.
- Rosenberg, A. H., and Gefter, M. L. (1969), *J. Mol. Biol.* 46, 581.
- Schweizer, M. P., Chheda, G. B., Baczynskyj, L., and Hall, R. H. (1969), *Biochemistry* 8, 3283.
- Thiebe, R., and Zachau, H. G. (1968), *Eur. J. Biochem.* 5, 546.
- Tomlinson, R. V., and Tener, G. M. (1962), *J. Amer. Chem. Soc.* 84, 2644.
- Wimmer, E., Maxwell, I. H., and Tener, G. M. (1968), *Biochemistry* 7, 2623.
- Yoshikami, D. (1970), Ph.D. Dissertation, Cornell University, Ithaca, N. Y.
- Yoshikami, D., Katz, G., Keller, E. B., and Dudock, B. S. (1968), *Biochem. Biophys. Res. Commun.* 166, 714.
- Yoshikami, D., and Keller, E. B. (1969), *Fed. Proc., Fed. Amer. Soc. Exp. Biol.* 28, 409.

On the Conformation of Lysozyme and α -Lactalbumin in Solution*

Eugene K. Achter† and Ian D. A. Swan

ABSTRACT: Lysozyme and α -lactalbumin have highly homologous primary sequences but different biological functions. W. R. Krigbaum and F. R. Kügler (1970, *Biochemistry* 9, 1216) have recently reported small-angle X-ray-scattering studies from both proteins in aqueous solution, and conclude that lysozyme and α -lactalbumin have quite different conformations in solution. They also present evidence for the

presence of α -lactalbumin dimer in solution. We demonstrate that all of the observed differences in small-angle X-ray scattering from α -lactalbumin and lysozyme in solution can be rigorously accounted for by such dimerization. Thus the experiments of Krigbaum and Kügler strongly suggest that these two proteins have quite similar conformations in solution.

Lysozymes cause cell wall lysis of gram-positive bacteria by a mechanism which is now fairly well understood (Phillips, 1966), while α -lactalbumin has been implicated in the lactose

* From the Laboratory of Molecular Biology, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland 20014. Received February 17, 1971.

lowered, it defined by

In cases now some agrees of the experim is thus equi of aggregat 1961) is univ equally well:

The extre the prese the small-an (1967) on m which gave permeation dimer

synthetase system (Ebner *et al.*, 1966). The primary sequences of hen egg white lysozyme and bovine α -lactalbumin have been shown to be strikingly similar, with regard to both residue identity (49 residues out of 123-129) and the positions of the disulfide bridges (Brew *et al.*, 1967, 1970). This homology is somewhat surprising in view of the differences in function, but does provide a basis for

- ¹ Bergman, J. W., and Maan, S. *South Int. Wheat Genet. Symp.*, Missouri, 329-335 (1973).
² Davis, J., *Ann. N.Y. Acad. Sci.*, 127 (1964).
³ Davidson, E. H., and Britten, R. J., *Rev. Biol.*, 48, 565-606 (1973).
⁴ Schwarz, D., *Genetics*, 67, 411-425 (1970).

RUDKIN et al

High resolution detection of DNA-RNA hybrids *in situ* by indirect immunofluorescence

We describe here a new method for the detection of RNA-DNA hybrids in cytological preparations with which we have revealed the locations of hybrid molecules on polytene chromosomes. The critical reagent is an antiserum raised in rabbits against poly(rA)-poly(dT) complexed with methylated bovine serum albumin, originally described by Stollar¹. The specificity and resolving power of the indirect immunofluorescence procedure are demonstrated using *in situ* hybridisation of 5S rRNA (ribosomal RNA) to polytene chromosomes of *Drosophila melanogaster* as a model system. The method has significant advantages over the autoradiographic procedures²⁻⁴ used so far.

The procedure for visualising the *in situ* hybrids follows Alfageme *et al.*⁵ It consists of exposing the cytological preparation to the rabbit anti-hybrid antiserum, then to anti-rabbit IgG prepared in goat and tagged with rhodamine, followed by examination in a fluorescence microscope (see legend to Fig. 1). Our test objects were polytene chromosomes of *Drosophila melanogaster* (giant phenotype) to which 5S rRNA had been hybridised *in situ* (see legend to Fig. 1). The two glands from a single larva either were used as duplicate samples or one gland served as a 'control' for the other. The preparations were not air dried at any time during the procedures. Test slides which were air dried at some stage before the immunological reactions were inferior to their controls either in the morphology of the chromosomes, the background fluorescence levels, the uniformity of the fluorescence staining or a combination of defects.

In situ hybridisation followed, in general, the recipe of Pardue and Gall⁶ with modification by Alonzo *et al.*⁷ (see legend to Fig. 1). The reaction was effective with 1.0 µg and with 0.2 µg 5S rRNA per slide. It is likely that much smaller amounts could be used if applied in a smaller volume and, perhaps, for longer times⁷⁻⁹.

The specificity of the immunological reagents in the cytological reaction is demonstrated by the confinement of chromosomal fluorescent label to the 56F region when 5S rRNA is included in the *in situ* hybridisation medium (Fig. 1) and by the absence of chromosomal fluorescence when 5S rRNA is omitted (not shown; would be black). Further evidence that the anti-hybrid antibodies are responsible for the chromosomal site of the positive fluorescence reaction is provided by the absence of fluorescence in chromosomes to which 5S rRNA had been hybridised but which were treated with antiserum absorbed with poly(rA)-poly(dT) (5 µg poly(rA)-poly(dT) per µl serum for 24 h at 4°C, centrifuged for 10 min at 6,000g). Thus the immunological reagents revealed only DNA-RNA hybrids within the nuclei of polytene cells prepared for *in situ* hybridisation.

The specificity was demonstrated further by analyses of the immunological properties of the antiserum. As described previously, several-thousand-fold dilutions of serum reacted in complement fixation assays with poly(rA)-poly(dT), poly(I)-poly(dC) or hybrids of natural RNA and DNA (ref. 1). A 1/50 serum dilution did not react with any single-stranded form of RNA or DNA or with double-stranded RNA or native DNA. When serum was assayed undiluted in counterimmunoelectrophoresis, weak reactions were seen with poly(rA) and with denatured DNA. Both these reactions were eliminated when the serum was passed through a poly(rA) Sepharose affinity column prepared as described by Poonian *et al.*¹⁰ (Fig. 3). With absorbed serum, which gave the same immunofluorescence as unabsorbed

serum, the hybrid was the only reactive polynucleotide class even in assay with undiluted serum.

There is a variable amount of fluorescence in cytoplasmic components, the origin of which is not yet known. Experimentally projected to attempt to block it while leaving the activity against hybrid nucleic acids intact. Occasional pale fluorescence observed in nucleoli is attributed to contamination of the 5S rRNA probe with fragments of 18S and 28S nucleolar rRNA.



Fig. 1 5S rRNA genes revealed in polytene chromosome 2R of *Drosophila melanogaster* by indirect immunofluorescence detection of RNA-DNA hybrids formed *in situ*. The two homologous 2R chromosomes are not paired except in their most distal portions. The 5S genes (56F on Bridges' standard map) are in the unpaired portions, those derived from one parent to the right, the other parent to the left. In each homologue at least two fluorescent cross-bands are visible. Arrows indicate the 56F regions in the upper photograph of the same chromosomes taken after staining with aceto-orcein. A salivary gland from a fully grown larva of *D. melanogaster* (giant phenotype) was fixed in 50% (v/v) aqueous acetic acid and squashed under a siliconed cover glass, then the slide was frozen on solid CO₂. After snapping off the cover slip, the slide was post-fixed in 3:1 ethanol:acetic acid (v/v), rinsed twice in 95% aqueous ethanol (v/v) and stored in 95% ethanol until used. Subsequent treatments were carried out in a moist chamber consisting of a 90-mm square culture dish containing a few leaves of bibulous (or filter) paper saturated with the solvent and two plastic strips to raise the slides above the wet paper. The reagent was placed between the slide and a cover slip. For hybridisation *in situ*, slides were first treated with pancreatic ribonuclease, 100 µg ml⁻¹ of 2-SSC (SSC is 0.15 M NaCl and 0.015 M sodium citrate) for 2 h at 25°C, then with 90% formamide in 0.1-SSC for 2 h at 65°C followed by ice cold 0.1-SSC rinses. The hybridisation reaction was carried out for 22-24 h at 37°C in 50% formamide in 4-SSC using either 1.0 or 0.2 µg 5S rRNA per slide. Highly purified 5S rRNA, extracted from *D. melanogaster* Oregon R embryos¹¹, after the annealing reaction, the slides were treated with pancreatic ribonuclease (15 µg ml⁻¹) in 2-SSC for 2 h at 25°C, rinsed with phosphate-buffered saline (PBS, 0.14 M NaCl, 0.01 M phosphate, pH 7.2) and exposed for 2 h at room temperature (21-23°C) to rabbit anti-DNA-RNA hybrid serum reconstituted from a lyophilised state by solution in water and diluted for use in PBS (1:20 for Fig. 1). After thorough rinsing in PBS, the slides were finally exposed to a rhodamine-labelled goat IgG fraction of anti-rabbit IgG (Miles-Yeda) reconstituted to approximately its original concentration, then diluted in PBS for use (1:20 for Fig. 1). The photographs were taken with a microscope equipped with a Zeiss epi-illumination fluorescence module using a 546-nm excitation filter, a 580-nm chromatic splitter and a 580-nm barrier filter for rhodamine fluorescence on 35-mm Eastman Tri-X Pan film exposed at ASA 1600 (Difline developer) at magnifications of approximately 150 (×40 objective) or 370 (×100 oil immersion objective). Fluorescence exposures were in the range of 1-8 s and phase contrast illumination was adjusted to require approximately the same exposure time so that a single frame could be exposed in both modes simultaneously (not shown here). In some instances, slides were stained with aceto-orcein after fluorescence photography had been completed, then photographed through a No. 58 filter (green) on Plus-X-Pan film at ASA 400. Approx. 1,000.

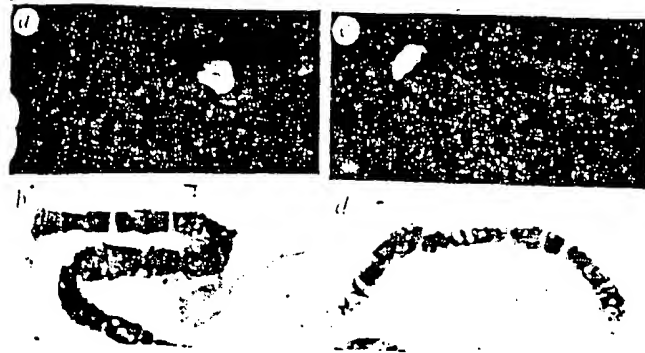


Fig. 2 5S rRNA genes revealed in *D. melanogaster* polytene chromosome 2R as in the legend to Fig. 1. The two chromosomes are unpaired in a single nucleus. The upper chromosome (a and b) is associated with the nucleolus (nu) at the locus of the 5S genes, fluorescent in (a). The homologous chromosome (c and d) is not visibly associated with the nucleolus; its 5S locus, indicated by the fluorescence in (c), is less discrete than that in its homologue (a). (b and d) Photomicrographs of the same fields as in (a) and (c), respectively, after staining with aceto-orcein. $\times 630$.

but the possibility that 5S DNA templates occur in nucleoli cannot be excluded.

The spatial resolving power of the immunofluorescent probe is equal to that of the optical system used to observe it. Autoradiographs can at best reveal a cluster of silver grains adjacent to, or covering, a labelled region which results in a resolving power of the order of 1–3 μm . So far the precise localisation of the 5S genes within the 56E/F region has been equivocal for *D. melanogaster* using autoradiography¹⁰. The images we obtain are of two kinds. In some nuclei, the fluorescence is restricted to a relatively narrow transverse 'band' which is often clearly made up of two subunits and is localised in the 56E region, distal to the puff usually present in 56E (Fig. 1). The possibility that the two subunits reflect the organisation of the 5S locus into the two separable sets of repeated sequences recently reported by Procnier and Tartof¹² is under investigation. On the other hand, the 5S region of chromosome 2R sometimes sticks to the nucleolus as in Fig. 2 (ref. 9) or is ectopically paired

to other chromosome regions. In those cases, the fluorescence may be distributed in a network of fibrils extending longitudinally along a much longer segment of chromosome (Fig. 2a). When that is true, the morphology in phase contrast and/or after post-staining with aceto-orcein is atypical in that the subsections 56E and F cannot be clearly demarcated and the region does not appear to be organised into distinct bands. Such dispersion of the *in situ* hybrids into fibrils is consistent with the suggestion of Steffensen and Wimber¹³ that the 5S genes may have been active in those chromosomes¹³. But the possibility that our 5S probe contains traces of contaminating nucleolar rRNA fragments that could reveal nucleolar rDNA adhering to chromosome 2 has not been entirely excluded.

The sensitivity of the technique has not yet been fully explored. At serum dilutions of 1 : 40 for the anti-hybrid rabbit serum and an equivalent concentration for the fluorescent reagent, the fluorescence intensity in the 5S region was very high. Photographic images should still be easily recordable at brightnesses one to two orders of magnitude lower. Thus, the possibility to detect the hybridisation of RNA copies of a unique gene in a polytene chromosome appears to be real. On the other hand, genes present in a size and multiplicity equivalent to the 5S of *D. melanogaster* may be detectable in uninemic chromosomes. An attempt is in progress to detect the 5S locus in human chromosomes at the pachytene stage of meiosis.

The technique is being used to study the distribution of naturally occurring chromosomal RNA detected as hybrid molecules. Polytene chromosomes mounted out of 50% acetic acid display a pattern of fluorescent regions when treated only with the immunological reagents. The regions do not fluoresce if the anti-hybrid serum is blocked with poly(rA)-poly(dT) or if the chromosomes are treated to remove indigenous chromosomal RNA (as in preparation for *in situ* hybridisation), indicating that they are sites of hybrid molecules. Since the locations of the sites change during larval and prepupal development, their RNA moiety could be involved with the control of transcription or of replication or both.

We thank Dr L. Cohen for bringing us together, Dr K. D. Tartof for highly purified *D. melanogaster* 5S RNA, Dr C. R. Allageme for rhodamine-tagged goat anti-rabbit IgG and for the use of a Zeiss epi-illuminator fluorescence attachment, all three colleagues for useful discussions and technical advice, and Miss D. J. Hazler for technical assistance. This work was supported in part by grants from the NSF and NIH and by an appropriation from the Commonwealth of Pennsylvania.

GEORGE T. RUDKIN

Institute for Cancer Research,
Fox Chase Cancer Center,
Philadelphia, Pennsylvania 19111

B. D. STOLLAR

Department of Biochemistry,
Tufts University School of Medicine,
Boston, Massachusetts 02111

Received October 18; accepted December 14, 1976.

- ¹ Stollar, B. D., *Science*, **189**, 609–611 (1970).
- ² Gall, J. G., and Pardoll, M. E., *Proc. natn. Acad. Sci. U.S.A.*, **64**, 600–604 (1969).
- ³ John, S. H., Birnstiel, M. L., and Jones, K. W., *Nature*, **223**, 582–587 (1969).
- ⁴ Henig, W., in *Int. Rev. Cytol.*, 36 (edit. by Boone, G. H., and Danielli, J. F.), 1–44 Academic, New York, 1973.
- ⁵ Wimber, D. E., and Steffensen, D. M., *J. Rep. Genet.*, **7**, 205–223 (1973).
- ⁶ Allageme, C. R., Rudkin, G. T., and Cohen, L. H., *Proc. natn. Acad. Sci. U.S.A.*, **73**, 2038–2042 (1976).
- ⁷ Pardoll, M. E., and Gall, J. G., in *Methods in Cell Biology*, 10 (edit. by Prescott, D. M.), 1–16 Academic, New York, 1975.
- ⁸ Alonzo, C., Helmsing, P. J., and Berendes, H. D., *Exp. Cell Res.*, **85**, 483–490 (1974).
- ⁹ Steffensen, D. M., and Wimber, D. E., in *Results and Problems in Cell Differentiation*, 3 (edit. by Ursprung, H.), 47–63 Springer, Wien, 1972.
- ¹⁰ Procnier, M. S., Schlachet, A. J., and Weissbach, V., *Biochemistry*, **19**, 424–427 (1976).
- ¹¹ Procnier, M. S., and Tartof, K. D., *Chromosoma*, **55**, 1–12 (1976).

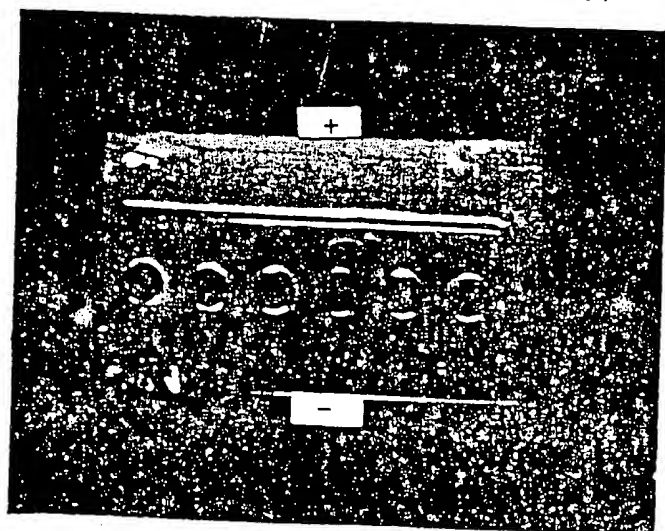


Fig. 3 Specificity of the absorbed antiserum. A sample of 7 ml of anti-poly(rA)-poly(dT) antiserum was absorbed by passage through a 1.2-ml column of poly(rA) Sepharose equilibrated with 0.1 M NaCl, 0.01 M phosphate, pH 7.2. The undiluted absorbed serum (200 μl) was placed in the trough; the wells contained 0.5 μg polynucleotide in 50 μl running buffer (0.05 M Tris-HCl, pH 8). The polynucleotides were, from left to right: poly(rA); denatured DNA; poly(rA)-poly(dT); poly(dT)-poly(rA); and poly(dA). The gel medium was 0.8% agar (Difco, purified agar) in running buffer; 7 ml of gel was poured on each 2–3 inch glass plate. Electrophoresis was run at 220 V

INTRODUCTION OF A FLUORESCENT LABEL AT THE 3'-OH END OF DNA AND
THE 3'-OH END OF THE GROWING RNA CHAIN

T. A. Rozovskaya, R. Sh. Bibilashvili,
N. B. Tarusova, G. V. Gurskii,
and S. A. Strel'tsov

UDC 547.963.3

Fluorescent-labeled 3'-(2')-O-acyl derivatives of uridine triphosphate were synthesized. The fluorescent component was introduced into the acyl residue. The optical properties of the fluorescent-labeled substrates were investigated. It was shown that calf thymus terminal deoxyribonucleotidyl transferase is capable of irreversibly adding a modified substrate to the 3'-OH end of DNA. In this case the concentration of free 3'-OH ends of DNA decreases exponentially as a function of the time of introduction of the modified substrate. The fluorescent-labeled analog of uridine triphosphate is also capable of being incorporated into the 3'-OH end of the growing chain of RNA by DNA-dependent RNA polymerase of *E. coli*. Incorporation of the analog inhibits DNA synthesis and leads to a decrease in the ability to synthesize RNA with time.

To study the mechanism of the action of RNA polymerase it seems necessary for the end of the growing chain of RNA and DNA protected from the action of DNase by RNA polymerase to contain paramagnetic or fluorescent groups. Especially interesting is the case of the introduction of two fluorescent labels, permitting an investigation of the resonance migration of energy.

Such a formulation of the problem determines the method of introduction and chemical structure of the label. Fluorescent-labeled 3'-(2')-O-acyl derivatives of uridine triphosphate were selected as the fluorescent label. Such a selection of the fluorescent label permits us to hope to introduce it into the growing chain of RNA using RNA polymerase itself. But protection of the 3'-OH group of ribose should ensure termination of the strand and, consequently, a priori localization of the label close to or within the active site of the enzyme.

The same substrate can be used to introduce a fluorescent label at the 3'-OH end of DNA fragments with the aid of calf thymus terminal deoxyribonucleotidyl transferase. The selection of the fluorescent groups themselves is determined by the value of the critical distance R_0 without emissionless migration of energy.

The present work is devoted to a study of the possibility of the incorporation of compounds of this kind by terminal transferase into the 3'-OH end of DNA and by RNA polymerase into the 3'-OH end of the growing RNA strand and to a determination of the kinetic parameters of the corresponding reactions.

METHODS

The UV spectra were taken on a Specord UV-Vis instrument (German Democratic Republic). The spectra of excitation and fluorescence were measured on a Hitachi spectrophotometer

Institute of Molecular Biology, Academy of Sciences of the USSR, Moscow. Institute of Automation and Control Processes, Far-Eastern Scientific Center, Academy of Sciences of the USSR, Vladivostok. Translated from Molekulyarnaya Biologiya, Vol. 11, No. 3, pp. 598-610, May-June, 1977. Original article submitted July 13, 1976.

(Japan) in a 4 mm cylinder 1 cuvette at a concentration of the carrier $\sim 10^{-7}$ M. Chromatography and electrophoresis were conducted on FN18 paper (German Democratic Republic), using the following systems: A: chloroform-ethanol-acetic acid (9:1:1.5); B: 1-butanol-water-acetic acid (5:3:2); C: 6% acetic acid, pH 2.5; D: 0.01 M solution of ammonium bicarbonate, pH 8. The electrophoretic mobility E_f^X was determined relative to compound X: glycine, picric acid (Picr), or histidine.

Preparations. Tris-HCl buffer, containing 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 5% glycerol, 0.5 mM β -mercaptoethanol, and Tris-acetate buffer, containing 50 mM Tris-CH₃COOH, pH 6.5, 30 μ M ZnSO₄, and 0.5 mM β -mercaptoethanol, were used. When necessary, MgCl₂ was added to these buffers to a concentration of 10 mM (Tris-HCl buffer) and 4 mM (Tris-acetate buffer).

The standard incubation solutions (0.25 ml) contained: 50 mM Tris-CH₃COOH, pH 6.5, 4 mM MgCl₂, 30 μ M ZnSO₄, 1 mM CoCl₂, 2 μ g terminal deoxyribonucleotidyl transferase, 5.2 μ g denatured DNA, 1 mM β -mercaptoethanol, deoxynucleoside triphosphates (solution 1); 50 mM Tris-HCl, pH 7.0, 10 mM MgCl₂, 10^{-1} mM EDTA, 5 mM β -mercaptoethanol, 50 mM KCl, 20 μ g native DNA, 10 μ g RNA polymerase, and ribonucleoside triphosphates (solution 2).

Cattle DNA (Olaïne Chemical Reagents Factory, USSR) and T₂ phage were purified by a phenol method [1], treated with RNase and pronase to remove RNA and proteins, and the treatment with phenol was repeated. The molecular weight of DNA was $5 \cdot 10^6$ and $30 \cdot 10^6$ daltons, respectively; the hyperchromic effect at 260 nm in thermal denaturation was ~ 27 and 37%.

Heat-denatured DNA was produced by heating DNA at 100°C for 5 min in the buffer 0.1 \times SSC, pH 7.0, in a concentration of 20 μ g/ml, followed by pouring the solution out onto a glass surface, cooled to the temperature of liquid nitrogen. The ratio of the volume of the liquid to the surface did not exceed 0.2 mm. The residual hyperchromism did not exceed 5% of the initial value.

Pronase (Serva, German Federal Republic) was used without additional purification. A solution of pronase (2 mg/ml) in Tris-HCl buffer without MgCl₂ was heated before use at 37°C for 2 h. The proteolytic activity in this case dropped by approximately 20-30%. The proteolytic activity of the enzyme was determined according to the rate of decrease in the amplitude of the circular dichroism of the substrate (bovine serum albumin) at 220 nm. During storage (at -10°C) the activity of the enzyme was unchanged in six months.

Deoxyribonucleotidyl transferase was isolated from the calf thymus according to the method of Chang and Bollum [2]. The specific activity of the enzyme was 10,000 units/mg. The incorporation of 1 nmole of the substrate into the acid-insoluble material in 60 min of incubation at 37°C in a standard incubation system was taken as the activity unit. The work of the enzyme was monitored according to the kinetics of the incorporation of deoxyribonucleoside triphosphate, labeled with tritium.

RNA polymerase was isolated from *E. coli* according to the method described earlier [3]. The specific activity of the enzyme was usually 0.4 ± 0.1 unit/mg. The incorporation of 1 μ mole of the substrate into the acid-insoluble material in 1 min of incubation at 25°C was taken as the activity unit of the enzyme. The radioactivity was measured on an SL-40 liquid scintillation counter (Intertechnique, France). During storage, the activity of the enzyme was practically unchanged.

Both enzymes did not contain determinable impurities of DNase, RNase, and proteolytic activity and were usually stored for 3-6 months at -10°C in a solution containing 30-50% glycerol and the standard incubation buffer without MgCl₂, in which β -mercaptoethanol was replaced by dithiothreitol in a concentration of 10^{-3} M.

Synthesis of Fluorescent-Labeled Analogs of the Substrate. Fluorescenyaminiothiocarbonylglycylglycine (compound I). To 1.5 g (3.4 μ moles) fluoresceny isothiocyanate, dissolved in 15 ml dimethylformamide, we added 0.95 g (7.2 μ moles) glycylglycine in 50 ml of 0.2 M carbonate-bicarbonate buffer, pH 9.0, mixed the solution for 4 h, acidified to pH 5 with acetic acid, diluted 1:2 with water, removed the precipitate, washed with 30 ml of water, dried, extracted with 50 ml ethyl acetate, and dried over NaOH. Yield 1.5 g (2.8 μ moles), 80%, calculated on the basis of the isothiocyanate. $\lambda_{\text{max}}^{\text{pH9}}$ 485 nm, R_f 0.3 (system A), E_f^{Gly} 0.17 (buffer C), E_f^{Picr} 1.1 (buffer D).

The dimethyl ester of I was produced by treating a solution of I in dimethylformamide with an ether solution of diazomethane, followed by isolation by electrophoresis on paper in buffer C and reprecipitation from a chloroform-ether mixture. E_f^{Gly} 0.8; E_f^{His} 0.77 (buffer C). Found: N 7.49%. Calculated for $\text{C}_{27}\text{H}_{33}\text{N}_3\text{O}_8$: N 7.7%.

3'(2')-O-Fluorescenylaminothiocarbonylglycylglyluridine-5'-triphosphate (compound III). To 450 mg (0.83 mmole) compound I, dissolved in 0.5 ml formamide, we added 162 mg (1 mmole) carbonyldiimidazole and mixed for 1.5 h at 20°C until the evolution of CO_2 stopped. The solution of the imidazole I obtained was added to 1.2 mmole UTP (substance II) in 0.2 ml formamide, mixed for 12 h at 6°C, after which it was diluted with 25 ml of acetone and centrifuged. The precipitate was dissolved in 1 ml of water, precipitated with 30 ml of alcohol, centrifuged, and the precipitate obtained dissolved in water. After separation by electrophoresis in buffer C (voltage gradient 22 V/cm), colored forms with E_f^{Picr} 0.5 and 0.43 were obtained. The first of them was eluted with 10 ml of water; the concentration of the substance was determined spectrophotometrically. $\lambda_{\text{max}}^{\text{pH9}}$ 454 nm. Yield 9.9%, calculated for compound II. R_f 0.55 in system B, E_f^{Picr} 0.5 in buffer C.

Hydrolysis of Compound III. Substance III is slowly hydrolyzed in aqueous solutions at pH 7, 8, and 9. Ammonia, ammonium bicarbonate, sodium bicarbonate, and carbonate-bicarbonate buffer were used as the base; aqueous alcohol solutions were also used. The rate of hydrolysis did not change significantly in these variations. When substance III was applied on a column with DEAE-cellulose, hydrolysis occurred rapidly. Eluting the column with an aqueous alcohol solution of ammonium bicarbonate, with variation of the concentration from 0 to 0.5 M, we successively eluted compound I and a mixture of uridine phosphates; the mole ratio of compound I to the mixture of nucleotides was 1:1 (determined spectrophotometrically according to the absorption intensity at 454 nm, pH 9, for substance I and at 260 nm, pH 2, for nucleotides).

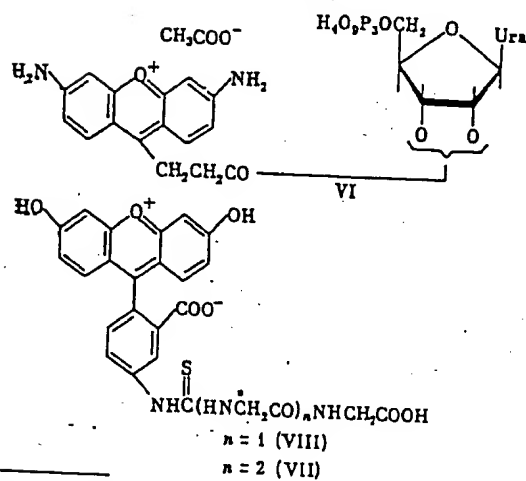
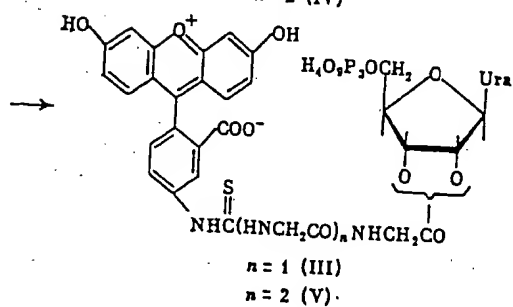
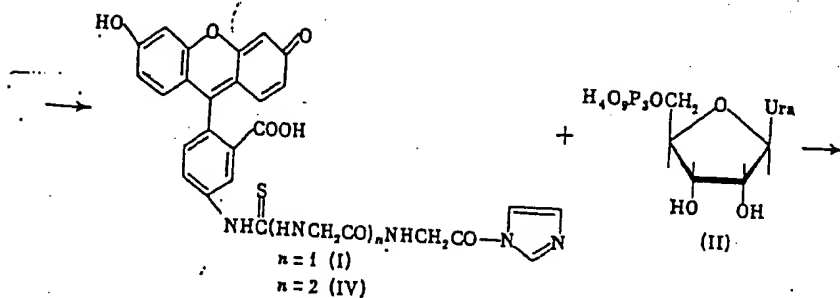
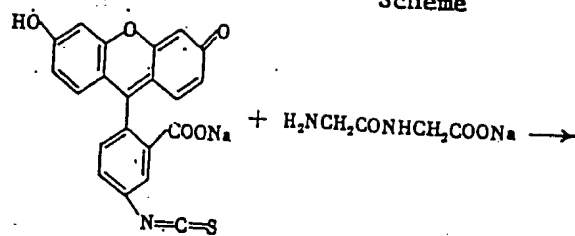
Fluorescenylaminothiocarbonyldiglycyl[^3H]glycine (compound IV). A 0.3 g portion (0.6 mmole) of compound I was dissolved in 2 ml of formamide, 0.11 g carbonyldiimidazole (0.7 mmole) was added, and the mixture mixed intensively for 1.5 h until the evolution of CO_2 ceased. The solution obtained was poured into 30 mg (0.4 mmole) [^3H]glycine (5.5 mCi/mmole) 5 ml of water and 0.8 ml 0.5 N NaOH, mixed for 2 h, after which the solution was acidified with acetic acid to pH 5, diluted to 50 ml with water, centrifuged, the precipitate washed with water (3×5 ml), and dried. Yield 170 mg, 51%. R_f 0.18 (system A).

3'(2')-O-Fluorescenylaminothiocarbonyldiglycyl[^3H]glycyluridine-5'-triphosphate (compound V). Substance V was obtained from compounds II and IV according to a method analogous to the method of production of compound III. Yield 10%. $\lambda_{\text{max}}^{\text{pH9}}$ 454 nm, R_f 0.5 in system B, E_f^{Picr} 0.5 (buffer C).

3'(2')-O-Rhodaminyuridine-5'-triphosphate (compound VI). Rhodamine S was preliminarily evaporated with 10 ml of 10% HCl and dried. A 70 mg portion (0.14 mmole) of rhodamine S was dissolved in 1 ml of formamide, 30 mg carbonyldiimidazole was added, and mixed for 15 min. The solution obtained was poured into 20 mg (0.037 mmole) UTP [compound (II)] in 0.2 ml formamide, mixed for 16 h at 6°C, precipitated with acetone, and centrifuged. The precipitate was dissolved in 1 ml of water, reprecipitated with 50 ml of alcohol, and again centrifuged, then dissolved in water and applied on paper. Electrophoresis was conducted in buffer C for 1.5 h. The colored zone with E_f^{Picr} 0.3 was eluted with 75 ml of water at 6°C. The concentration was determined spectrophotometrically at $\lambda_{\text{max}}^{\text{pH7}}$ 520 and 550 nm, R_f 0.4 in system B (plates with cellulose).

Hydrolysis of Compound VI. A 0.5 μmole portion of substance VI was dissolved in 10 ml of 0.2 M ammonium bicarbonate solution, evaporated at 30°C after 2 h, dissolved in water, and applied on a column with CM-cellulose. The nucleotides were eluted with water; rhodamine S with 5% acetic acid. The nucleotide concentration was determined spectrophotometrically at 260 nm, pH 2; rhodamine at 555 nm, pH 9. The ratio of nucleotides and rhodamine was 1:1. The time of half-hydrolysis of compound VI $\tau_{1/2}$ was determined spectrophotometrically. Hydrolysis was conducted in buffer with pH 8. The value of $\tau_{1/2}$ was 1 h. In hydrolysis in buffer with pH 10, $\tau_{1/2}$ for triphosphates was equal to 4 min, and for diphosphate 20 min.

Purification from Decomposition Products. In the case of storage even in the lyophilized state, fluorescent-labeled 3'(2')-O-acyl derivatives of uridine triphosphate were broken down forming numerous substances, among which uridine mono-, di-, and triphosphates, both containing the fluorescent group and without it, were found. The isolation and purification of the



† Denotes radioactively labeled compound VII.

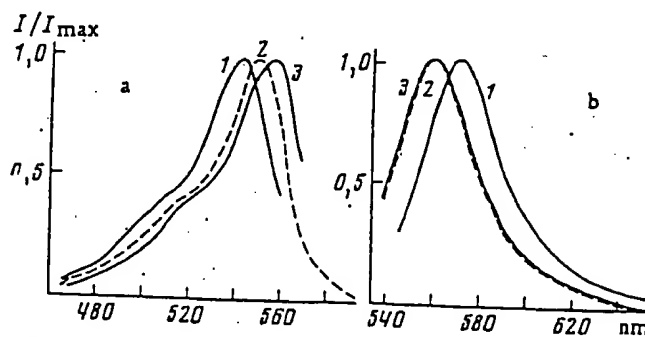


Fig. 1. Excitation (a) and fluorescence (b) spectra: 1) rhodamine S; 2) compound VI; 3) dA₁₀, labeled with compound VI.

fluorescent triphosphate was conducted using chromatography on a column with Dowex-50 in the Na⁺ form in 0.05 M sodium phosphate buffer, pH 6.0.

Nucleotides containing rhodamine were eluted with 0.5 M NaCl in the same buffer, containing 20% dioxane. The eluate was evaporated under vacuum at room temperature and chromatographed on a column with Sephadex G-15 in 0.005 M sodium phosphate buffer, pH 6.0. The first peak, containing the triphosphate, was evaporated under vacuum to a small volume and stored before use at -50°C. The content of di- and monophosphates was monitored by ascending chromatography of the purified preparation on DEAE-paper DE 81 in 0.5 M ammonium formate, pH 3.0, containing 50% dioxane.

RESULTS AND DISCUSSION

Fluorescent-labeled 3'(2')-O-acyl derivatives of uridine triphosphate were synthesized. The fluorescent component was introduced into the acyl residue. Derivatives of fluorescein and rhodamine S were selected as such a component. In the first case the starting material in the synthesis was fluoresceinylisothiocyanate, which was condensed with diglycine; the fluoresceinylaminothiocarbonylglycylglycine obtained in this way was converted to an imidazolide (compound I), which was reacted with uridine triphosphate (compound II). Compound III was obtained with a small yield (see Scheme). This is explained to a substantial degree by the partial destruction of triphosphate under the conditions of the reaction. The synthesis was conducted in formamide; substance III was practically not formed in an aqueous-organic mixture.

The interaction of the imidazolide (compound I) with [³H]glycine yielded compound IV, the imidazolide of which then acylated substance II with the formation of compound V. In a study of the properties of substances III and V, it was noted that the ester bond in them is rather stable; slow hydrolysis of it occurs in aqueous and aqueous alcohol solutions at pH 7, 8, and 9 at approximately the same rate. In the case of application on DEAE-cellulose, rapid hydrolysis was observed. The hydrolysis products of substances III and V were compounds I and IV, respectively, and a mixture of uridine mono-, di-, and triphosphates. This is an indication of the instability of the triphosphate portion of the molecule. Its partial destruction was also observed during storage of substances III and V.

Uridine triphosphate, acylated with the rhodamine S residue (compound VI), was produced analogously. The ester bond in this compound was already rather rapidly hydrolyzed at pH 7 and 8. The triphosphate component in this compound was also unstable. In one month of storage in the lyophilized state at -20°C, no more than 30% of the triphosphates were preserved. The excitation and fluorescence spectra for the uridine triphosphate analog containing rhodamine S and free rhodamine S are given in Fig. 1.

The excitation and fluorescence spectra for compound VII are shown in Fig. 2. The values of the molar extinction for fluorescent-modified analogs of uridine triphosphate, for free rhodamine S, and for free fluorescein are cited in Table 1.

The molar extinctions at pH 10 were measured in 0.1 M carbonate buffer. The molar extinctions of fluorescein, rhodamine S, and compound III were determined according to a weighed sample.

The molar extinction of compound VI was determined as follows: The absorption of a solution of preparation VI of arbitrary concentration was measured at pH 10 until it decomposed to uranyltriphosphate and free rhodamine S. Then, after complete breakdown of the substance, knowing the molar extinction of free rhodamine S, the concentration of free rhodamine S in the decomposed preparation was determined, i.e., the concentration of compound VI in the solution. Knowing the absorption of a solution of compound VI before its decomposition, we determined the molar extinction of preparation VI.

To determine the molar extinction of preparation V, the following measurements were performed. A radioactively labeled compound VII and compound V radioactively labeled in the same position with the same specific activity and a known value of the molar extinction for preparation VII were taken. An aliquot of a solution of known concentration of compound VII was counted on a liquid scintillation counter to determine the specific activity of the preparation. Knowing the absorption spectrum and specific activity of the radioactively labeled compound V, we determined its concentration in solution and the value of its molar extinction.

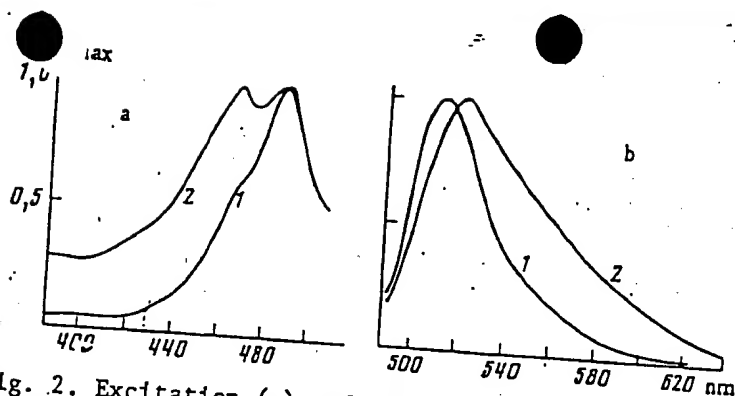


Fig. 2. Excitation (a) and fluorescence (b) spectra: 1) of compound VII; 2) of dT₁₀, labeled with compound V.

TABLE 1. Molar Extinctions of Fluorescent Analogs of the Substrates

Compound	ϵ	λ_{\max} , nm
V	12 600	452
III	37 000	485
Fluorescein	85 000	485
VI	65 000	549
Rhodamine S	66 000	543

The absorption spectra of fluorescently labeled analogs and rhodamine S depend greatly on the concentration, beginning with values $>10^{-6}$ M. At concentrations $<10^{-6}$ M, this dependence is negligible.

The spectrum of free rhodamine S does not depend on the pH value in the range of 6-10. The change in the shape of the spectrum of the substrate, modified with rhodamine S, and the shift of the maximum at pH 9-10 are related to the decomposition of the modified substrate to uranyl phosphate and rhodamine S, which begins at these pH values.

The spectrum of the substrate modified with fluorescein (compound V) does not depend on the pH in the region of 5-10. The absorption spectra of compounds III and free fluorescein depend greatly on the pH value in the range of 5.0-7.5. The stability of compounds III and V, in contrast to the analog modified with rhodamine S, does not depend on the pH value.

Incorporation of the Fluorescent Label into the 3'-OH End of DNA by Bollum's Terminal Transferase. Using fluorescent-labeled analogs of nucleotides in the synthesis of DNA with terminal deoxyribonucleotidyl transferase, we expected the formation of a product containing a blocked 3'-OH end, which would be inactive in further synthesis. In the presence of an excess of the enzyme, the rate of the reaction of elongation of DNA is determined by the concentration of free 3'-OH ends of DNA. In this case, if the enzyme is capable of irreversibly adding the modified substrate at the 3'-OH end of DNA, the concentration of free 3'-OH ends should show a consistent exponential decrease.

The working ratio of enzyme and DNA concentrations corresponds to the point A of the curve in Fig. 3 (the point A is the maximum value of the linear portion of the curve, where the concentrations of free 3'-OH ends is the factor determining the rate of the reaction of DNA elongation).

To measure the initial concentration of free 3'-OH ends of DNA we used the ability of the terminal transferase to add a limited number of ribonucleotides to the 3'-OH end of a polydeoxyribonucleotide [4, 5]. Under the conditions of the experiments, all the free 3'-deoxyribonucleotides were saturated with labeled ribonucleotides (Fig. 4). The product was subjected to alkaline hydrolysis, so as to exclude the formation of di- and triribonucleotides. The reaction was conducted as follows: To a standard incubation solution 1 we added [³H]UTP ($0.5 \cdot 10^{-3}$ M) with specific activity 1.7 Ci/mmole, incubated for 2 h at 37°, and subjected the reaction product to alkaline hydrolysis at pH 13.5 for 24 h at 35°C. The acid-insoluble

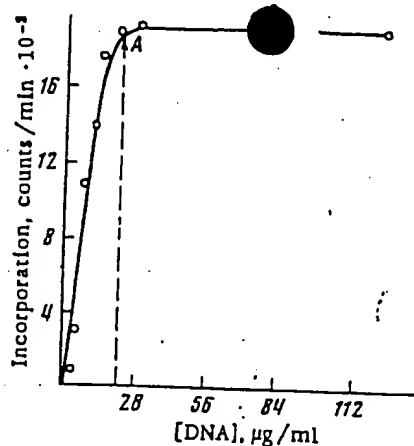


Fig. 3. Dependence of the rate of DNA synthesis on the initial concentration of the DNA primer (incubation solution 1; $0.5 \cdot 10^{-4}$ M $[^3\text{H}]\text{dATP}$ with specific activity 0.1 Ci/mmole; 40 min (37°)).

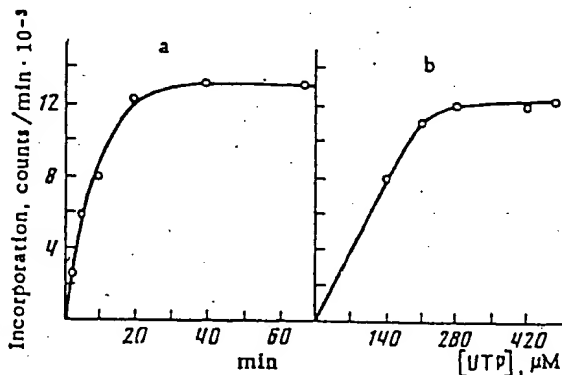


Fig. 4. Determination of the concentration of free 3'-OH groups in DNA: a) dependence of the incorporation of $[^3\text{H}]\text{UTP}$ on the time; b) dependence of the incorporation of single molecules of UTP at the 3'-OH end of the DNA primer on the substrate concentration.

fraction was collected on nitrocellulose filters and the radioactivity measured (Fig. 4b). A 2 h incubation is sufficient for saturation of all the free 3'-OH ends of DNA with UTP molecules (Fig. 4a), since the level of saturation is already reached after 4 min. The average length between single-stranded breaks in DNA was calculated from the ratio of the amount of $[^3\text{H}]\text{UMP}$ incorporated at 3'-OH end of the DNA fragment to the amount of DNA nucleotides in the sample (in moles). This ratio was equal to 1:800 for the DNA preparation used.

The effective Michaelis constant for UTP was measured from the dependence of the rate of incorporation of $[^3\text{H}]\text{UTP}$ with specific activity 1 Ci/mmole in 20 min of incubation at 37°C on its concentration. The value of K_m was of the order of $2 \cdot 10^{-4}$ M.

As has already been stated, in the case of irreversible incorporation of the modified substrate and the initial conditions described above, we had to expect an exponential decrease in the rate of incorporation of deoxynucleoside triphosphate as a function of the time of preincubation of the system with the UTP analog before the addition of deoxynucleoside triphosphates. It is precisely such reaction kinetics that is observed in the preincubation of terminal transferase and denatured DNA with fluorescent-labeled analogs of the substrate. The reaction was conducted as follows: Compound III to a concentration of $1.95 \cdot 10^{-5}$ M or substance VI (to $2.3 \cdot 10^{-5}$ M) or $1.95 \cdot 10^{-5}$ M UTP was added to the standard incubation solution 1. Incubation was conducted for some time at 37°C , then $[^3\text{H}]\text{dATP}$ with specific activity 0.15 Ci/mmole was added to a concentration of $6 \cdot 10^{-5}$ M. Incubation was conducted for 10 min at 37°C , and the reaction was stopped by adding EDTA. The results of the experiment are shown in Fig. 5. As is shown by the control curve, UTP does not block the 3'-OH end of DNA.

However, the curves in Fig. 5 may take this form, in the first place, if the enzyme is irreversibly inactivated during binding to the fluorescent-labeled analog and, in the second place, if the enzyme forms a nondissociated complex with DNA after incorporation onto the 3'-OH

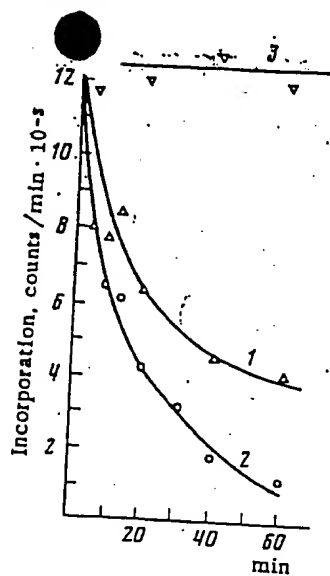


Fig. 5

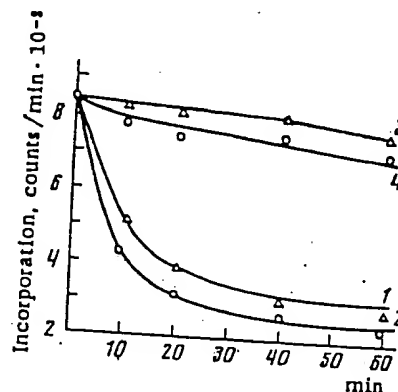


Fig. 6

Fig. 5. Kinetics of blocking of the 3'-OH ends of DNA by fluorescent analogs of UTP: 1) compound III, activity of incubation solution 13,000 counts/min; 2) compound VI, 13,000 counts/min; 3) UTP, 8500 counts/min (curves standardized according to the activity of the corresponding samples without addition of an inhibitor).

Fig. 6. Kinetics of blocking of the 3'-OH ends of DNA by fluorescent analogs of substrates: 1 and 2) DNA concentration 21 $\mu\text{g/ml}$; 3 and 4) 100 $\mu\text{g/ml}$; 2 and 4) concentration of compound VI $2.5 \cdot 10^{-3}$ M; 1 and 3) concentration of compound III $2.5 \cdot 10^{-3}$ M.

end of the DNA. In order to exclude both these possibilities, an experiment was conducted analogous to the preceding one, but with two different DNA concentrations, selected so that in one case the reaction of addition of deoxyribonucleotides limits the concentration of 3'-OH ends of DNA and, on the other hand, the enzyme concentration. As can be seen from the data of Fig. 6, no inactivation of the enzyme occurs, since when the DNA concentration is not the rate-limiting factor, the rate of addition of deoxynucleotides to DNA practically does not decrease after preincubation with analogs of uridine triphosphate.

To determine the effective Michaelis constant for modified analogs of uridine triphosphate, we measured the dependence of the rate of blocking of the 3'-OH ends of DNA by these compounds on their concentration. The scheme of the experiment is analogous to the schemes of the two preceding experiments. A comparison of the effective Michaelis constants for fluorescent-labeled analogs of uridine triphosphate and for uridine triphosphate itself shows that despite the presence of fluorescent groups in analogs of the substrate and the positive charge of the fluorescent group in compound VI, the analogs of the substrate and the substrate itself are incorporated at the 3'-OH end of DNA at rates and effective Michaelis constants of equal magnitude.

It is curious to note that analogous experiments both with 2'-O-Me-ATP and with 3'-O-Me-ATP, synthesized as described earlier [6], did not lead to blocking of the 3'-OH ends, either in DNA or in the oligonucleotide dT₁₀.

Let us note that the curves of blocking of the 3'-OH ends, modified with the substrate, in general for long times deviate from exponential functions. Probably this is due to the insufficient stability of the fluorescent-labeled substrate. Possibly, during the work, part of the UTP analog loses its fluorescent label. Then part of the previously blocked 3'-OH ends of DNA again become accessible to further lengthening of the DNA strand. To demonstrate the incorporation of a fluorescent label into the 3'-OH end of DNA, we conducted the following experiment: dT₁₀ in a concentration of 10^{-4} M was incubated with the terminal transferase in a concentration of $2 \cdot 10^{-3}$ M in buffer containing 5 mM MgCl₂, 1 mM CoCl₂, 100 mM sodium cacodylate, pH 7.0, and 0.3 mM fluorescent-labeled analog. Incubation was conducted at 37°C.

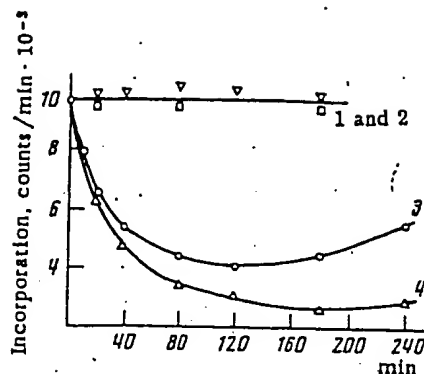


Fig. 7

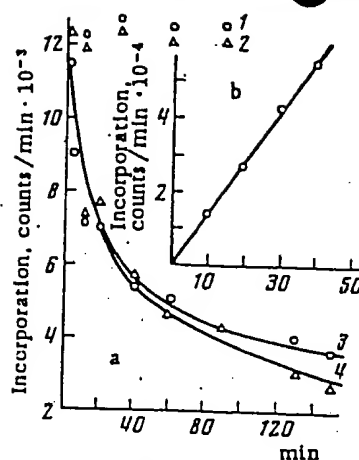


Fig. 8

Fig. 7. Kinetics of the blocking of the 3'-OH ends of dT₁₀: 1) 3'-O-Me-ATP, 2) UTP, 3) compound VI, 4) compound V.

Fig. 8. Kinetics of the incorporation of fluorescent analogs of the substrates into the 3'-OH end of the growing chain of RNA. a) Incubation in the presence of 4 nucleoside triphosphates (3 and 4) and in their absence (1 and 2). 1 and 3) Compound VI; 2 and 4) compound III. b) Dependence of the rate of synthesis on the time.

After definite time intervals, aliquots with a volume of 2 μ l were collected and transferred to 20 μ l of fresh incubation medium with the same buffer and the same enzyme concentration, but not containing oligonucleotides, and containing 0.2 mM [³H]dATP with specific activity 0.02 Ci/mmol in place of UTP analogs.

After 10 min of incubation at 25°C, the sample was applied on DEAE-paper DE 81 and chromatographed in 0.5 M ammonium formate, pH 3.0, in a 50% solution of dioxane in water. The oligonucleotide dT₁₀, together with the [³H]dAMP bonded to it, remained at the start. The chromatograms were dried, cut into uniform pieces, and the radioactivity analyzed in a liquid scintillation counter. The amount of [³H]dATP bonded to dT₁₀ is shown in Fig. 7 as a function of the time of incubation of the system with ribonucleoside triphosphate analogs.

After 2.5 h of incubation, the bulk of the incubation medium (100 μ l) was applied on a column of G-50 (14 ml, 0.5 × 70 cm), equilibrated with 0.01 M potassium phosphate buffer, pH 6.0, and chromatographed at a rate of ~2 ml/h. The first peak containing the oligonucleotide was lyophilized, dissolved in 0.1 ml of water, and deproteinized by shaking with a mixture of phenol, chloroform, and isoamyl alcohol; the aqueous phase was subjected to chromatography three times on a G-50 column. In the last chromatography, an almost complete coincidence of the elution profiles measured according to the absorption at 260 nm and the fluorescence was observed, with the absence of a peak in the region of the low-molecular-weight substrate, not bound to dT₁₀. This result demonstrates the incorporation of the modified substrate into the 3'-OH end of dT₁₀. An analysis of the content of fluorescein and rhodamine S in an analogous experiment with dA₁₀, as well as radioactive glycine in an oligonucleotide with dT₁₀, showed that despite substantial inhibition of the reaction of the addition of dATP, observed directly after the reaction with the analog (Fig. 7), the fraction of oligonucleotides fluorescently labeled in the final product is negligible, and is ~7% for fluorescein-labeled dT₁₀, and 1% of all the oligonucleotides for rhodamine S-labeled dA₁₀. This difference is evidently explained by a constant loss of the label during purification and storage of the labeled oligonucleotides.

Incorporation of a Fluorescent Label into the 3'-OH End of RNA by RNA Polymerase. Just as in the case of terminal transferase, the incorporation of a uridine triphosphate analog, carrying a fluorescent label in the RNA strand, should lead to an exponential decrease in the rate of RNA synthesis with time. However, this condition is fulfilled only in the absence of initiation of RNA synthesis, i.e., in the case of apparent inactivation of enzyme molecules that have incorporated the analog. The experiment was conducted as follows: To a standard incubation solution 2 we added ATP, GTP, and CTP in a concentration of $8 \cdot 10^{-3}$ M

each, unlabeled UTP (10^{-3} M), and the fluorescently labeled UTP analog ($2.4 \cdot 10^{-5}$ M); after incubation for the time indicated in Fig. 8 at 37°C , [^3H]UTP was added to the incubation mixture (to a final concentration of $0.8 \cdot 10^{-5}$ M) with specific activity 1 Ci/ μmole , incubated for 10 min, and the reaction ended by the addition of EDTA. The result of this experiment is shown in Fig. 8a. The control experiment, excluding the possibility of apparent inactivation of the enzyme in the case of its direct bonding to the fluorescent-labeled analogs, was conducted as follows: The fluorescent-labeled substrate was incubated with RNA polymerase in standard solution II without ribonucleoside triphosphates; after incubation for a definite time (Fig. 8a), all the nucleoside triphosphates were added in the same concentrations as in the preceding experiment. After this, the solution was incubated for 10 min, and the reaction stopped. The absence of appreciable inhibition (Fig. 8a) of synthesis of RNA with such an experimental procedure shows that RNA polymerase is not inactivated by modified analogs of the substrate directly without RNA synthesis. The control kinetics of RNA synthesis, showing that when four nucleoside triphosphates are used in the preceding two cases, the synthesis of the product is a linear function of the time of the reaction, is depicted in Fig. 8b. The control dependence of the rate of RNA synthesis on the RNA polymerase concentration showed that the rate is directly proportional to the enzyme concentration (up to $160 \mu\text{g/ml}$).

On account of the insufficient stability of the fluorescent-labeled UTP analogs used, free rhodamine S or compound VIII may be found in the incubation system.

We investigated the influence of these compounds on the rate of RNA synthesis and the dependence of RNA synthesis on the time in the presence of rhodamine S or compound VIII. The results of the experiments showed that rhodamine S has practically no effect on RNA synthesis, while substance VIII somewhat stimulates it.

To demonstrate the incorporation of a fluorescent-labeled analog of the substrate into the 3'-OH end of the growing RNA chain, we conducted a preparative synthesis of RNA in the presence of the modified substrate.

Four ribonucleoside triphosphates in a concentration of $2 \cdot 10^{-3}$ M each and the fluorescently labeled UTP analog ($3.5 \cdot 10^{-6}$ M) were added to the incubation solution 2, containing $100 \mu\text{g}$ of native thymus DNA and $100 \mu\text{g}$ RNA polymerase. After 2 h of incubation at 37°C , the reaction product was chromatographed on a column of G-150 (10 ml, $0.5 \times 50 \text{ cm}$), equilibrated with 10 ml Tris-acetate buffer, pH 6.5, containing 0.1 M KCl. After the first chromatography, the peak of the high-molecular-weight fraction was collected, concentrated, treated with pronase, and rechromatographed.

In both cases, with the analog containing both rhodamine S and fluorescein, the content of the fluorescent label in the product of synthesis was more than three times as high as in the control experiments, considering the possible adsorption of the fluorescent-labeled analogs on the high-molecular-weight components of the system.

We undertook an attempt to measure the effectiveness of the transfer of energy of excitation between fluorescent-labeled analogs bound to a matrix.

In a preparation obtained by annealing of fluorescent-labeled dA_{10} and dT_{10} , a quenching of fluorescence was observed. However, the value of the quenching was very low. This is due to the fact that the probability that both the donor and the acceptor will be on a single double-stranded molecule is low. Attempts were undertaken to enrich preparations of dA_{10} labeled with rhodamine S through the reaction of elongation of dA_{10} molecules that do not contain rhodamine S on the 3'-OH end, using terminal transferase and separation of the lengthened and unlengthened oligonucleotides on Sephadex G-100. However, these attempts were unsuccessful as a result of the progressive loss of the fluorescent label during all the procedures.

In this work we demonstrated the theoretical possibility of incorporation of fluorescently labeled analogs of nucleotides by terminal transferase at the 3'-OH end of DNA and by RNA polymerase at the 3'-OH end of the growing RNA strand. However, on account of the insufficient stability of the fluorescently modified analogs of UTP used in the work it was difficult to work with them.

Noteworthy is a peculiarity of the reactions in which fluorescent-labeled analogs are substrates and, in addition, irreversible inhibitors, which terminate the chain of the

polymer being synthesized.

The kinetic constants of the reaction with analogs and with natural nucleoside triphosphates for the reaction of addition of ribonucleotides by terminal deoxyribonucleotidyl transferase proved extremely close. This does not depend on whether the enzyme is deficient in excess with respect to the primer, but it means that at least in the reaction of addition of ribonucleotides, the terminal transferase is freed from the complex with the primer after each stage of addition of the nucleotide to the primer.

It is interesting, moreover, that both analogs of nucleoside triphosphates, modified on the ribose both with uncharged (fluorescein) and with charged (rhodamine S) voluminous substituents, proved capable of being incorporated into the chain of the growing polymer. This property is inherent not only in the terminal deoxyribonucleotidyl transferase of the calf thymus and DNA-dependent RNA polymerase of *E. coli*, but also the two-component ribonucleotidyl transferase [7]. Analogous compounds 2'(3')-O-isovaleryl- and 2'(3')-O-(α -methoxyethyl)nucleoside diphosphates are good substrates for polynucleotide phosphorylase [8].

At the same time, such compounds as 2'-O-Me-ATP and 3'-O-Me-ATP, entirely incapable of blocking the growing ends of the polynucleotide in the reaction of calf thymus deoxyribonucleotidyl transferase, also proved to be only weak inhibitors of two-component ribonucleotidyl transferase of *E. coli* [7]. But for DNA-dependent RNA polymerase of *E. coli*, 3'-O-Me-ATP served as an inhibitor analogous in action to the fluorescent-labeled analogs of nucleoside triphosphates and 3-dATP, whereas 2'-O-Me-ATP did not inhibit the synthesis at all.

The data cited here show that the picture of the interaction of substrates with the enumerated enzymes is complex, and that not only steric, but also some other mechanisms participate in this process.

LITERATURE CITED

1. G. P. Georgiev, in: Nucleic Acids (ed. by I. B. Zbarskii and S. D. Debov) [in Russian], Moscow (1968), p. 74.
2. L. Chang and F. I. Bollum, J. Biol. Chem., 246, 909-916 (1971).
3. R. Sh. Bibilashvili and L. P. Savochkina, Mol. Biol., 7, 90-103 (1973).
4. H. Kössel and R. Roychoudhury, Eur. J. Biochem., 22, 271-276 (1971).
5. H. Kössel and R. Roychoudhury, Eur. J. Biochem., 22, 310-320 (1971).
6. S. M. Hecht, S. D. Hawrelak, and I. W. Kozarich, Biochem. Biophys. Res. Commun., 52, 1341-1347 (1973).
7. D. A. Prangishvili and R. Sh. Bibilashvili, Mol. Biol., 1, 203-216 (1977).
8. G. Kaufman, M. Fridkin, A. Zutra, and U. Z. Litthauer, Eur. J. Biochem., 24, 4-11 (1971).

MOLECULAR BIOLOGY

A translation of *Molekulyarnaya Biologiya*

December, 1977

Volume 11, Number 3, Part 2

May-June, 1977

CONTENTS

	Engl./Russ.
Introduction of a Fluorescent Label at the 3'-OH End of DNA and the 3'-OH End of Growing RNA Chain - T. A. Rozovskaya, R. Sh. Bibilashvili, N. B. Tarusova, G. V. Gurskii, and S. A. Strel'tsov	455 598
Suppression of the Inducible Synthesis of Enzymes in an <i>Escherichia coli</i> K12 Mutant with a Deleted ptsH Gene - V. N. Gershanovich, T. S. Il'inā, O. Yu. Rusina, N. V. Yurovitskaya, and T. N. Bol'shakova	466 611
Nuclear Ribonucleoprotein Particles Containing Messenger RNA. 12. Study of the Dissociation and Reconstruction of 30S Particles - V. V. Kul'guskin	474 620
Selection and Certain Properties of Recombinant Clones of λ Phage, Containing Genes of <i>Drosophila melanogaster</i> - Yu. V. Il'in, N. A. Churikov, A. S. Solonin, L. G. Polukarova, and G. P. Georgiev	489 637
Structure of Higher Plant Chloroplast Membranes as Studied by Paramagnetic Probes - E. K. Ruuge, V. K. Subchinskii, and A. N. Tikhonov	496 646
Effect of Proteins Bound to Single-Stranded DNA on the Synthesis of RNA and Poly(A). III. The Protein Product of Phage ϕ Gene 5 - Yu. S. Polonskii, N. M. Naidenova, Yu. I. Kozlov, Yu. N. Zograf, and V. G. Debabov	504 656
Molecular Mechanisms of Spontaneous Transversions and Transitions - V. I. Poltev and V. I. Bruskov	509 661
Investigation of the Enzymatic Reaction Catalyzed by <i>E. coli</i> Ribosomes - V. V. Kotusov, M. K. Kukhanova, N. E. Sal'nikova, L. V. Nikolaeva, A. A. Kraevskii, and B. P. Gottikh	517 671
Electron Microscopic Investigation of Chromatin in Hepatocyte Nuclei in situ during the First Hours after Partial Hepatectomy - V. M. Manteifel', Yu. V. Ershov, I. N. Bandrina, and A. V. Zelenin	523 677
Measurement of the Time of Rotational Correlation of Macromolecules by the ESR Method in the Case of Covalently Bound Spin Label - I. V. Dudich, V. P. Timofeev, M. V. Vol'kenshtein, and A. Yu. Misharin	531 685
Nomograms for Determining the Sedimentation Constants of Nucleic Acids by Centrifuging in a Sucrose Concentration Gradient on Spinco Ultracentrifuges - L. A. Osterman	539 694
Influence of Histone Kinase, Phosphorylating Lysine-Rich Histone Fractions, Normal State and after Partial Hepatectomy - E. A. Kir'yanova, V. A. Kolésnikov, M. V. Nesterova, E. S. Severin, and A. V. Zelenin	550 706

The Russian press date (podpisano k pechatl) of this issue was 4/15/1977. Publication therefore did not occur prior to this date, but must be assumed to have taken place reasonably soon thereafter.

FORTSCHRITTE
DER CHEMIE ORGANISCHER
NATURSTOFFE

PROGRESS IN THE CHEMISTRY
OF ORGANIC NATURAL PRODUCTS

BEGRÜNDET VON · FOUNDED BY
L. ZECHMEISTER

HERAUSGEGEBEN VON · EDITED BY

W. HERZ H. GRISEBACH G. W. KIRBY
TALLAHASSEE, FLA. FREIBURG I. BR. GLASGOW

VOL. 32

VERFASSEN · AUTHORS

R. J. HIGHET · H. KÖSEL · P. G. SAMMES
P. M. SCOPES · W. K. SEIFERT · H. SELIGER
E. A. SOKOLOSKI · H. C. VAN HUMMEL



1975

WIEN · SPRINGER-VERLAG · NEW YORK

Recent Advances in Polynucleotide Synthesis

By H. KÖSSEL, Institut für Biologie III der Universität
Freiburg i. Br., Federal Republic of Germany, and
H. SELIGER, Institut für Makromolekulare Chemie der Universität
Freiburg i. Br., Federal Republic of Germany

Contents

Introduction	298
Abbreviations and Symbols	299
I. Protecting Groups	300
1.1. General Considerations	300
1.2. Choice of Blocking and Deblocking Conditions	304
1.3. Survey of Blocking Groups	305
1.3.1. Protecting Groups for the Phosphate Moiety	346
1.3.2. Protecting Groups for the Hydroxyl and Amino Functions of the Sugar and Base Moieties	348
1.3.3. Protecting Groups for the Vicinal Diol Group of Ribonucleic Acid Con- stituents	352
1.4. Protecting Groups with Special Applications	353
1.4.1. Protecting Groups for Solvent Extraction	353
1.4.2. Protecting Groups for Separations by Affinity Chromatography	354
1.4.3. Activable Protecting Groups	355
1.5. Strategy of Consecutive Blocking or Deblocking of Several Functions	356
1.6. General Blocking Schemes for Intermediates of Polynucleotide Synthesis	357
2. Phosphorylation Methods in the Synthesis of Mono- and Oligonucleotides	367
2.1. Transfer of a Phosphoryl Group	368
2.2. Transfer of a Phosphate Group	393
2.3. Miscellaneous Chemical Phosphorylation Reactions	394
2.3.1. Phosphorylation by Oxidation of Nucleoside Phosphites	395
2.3.2. "Thermal" Phosphorylation	396
2.3.3. Prebiotic Phosphorylations	397
2.4. Enzymic Phosphorylation	397
3. Separation Techniques	401
3.1. Column Procedures	401
3.1.1. Column Chromatography on Conventional Adsorbent Types	401
3.1.2. Column Chromatography on Newly Developed Adsorbent Types	404

3.2. Extraction Procedures	405
3.3. Miscellaneous Techniques	406
4. Formation of Internucleotide Linkages by Chemical Synthesis	406
4.1. Conventional Methods	406
4.1.1. Synthesis in the Deoxy Series	406
4.1.2. Synthesis in the Ribo Series	406
4.1.3. Modified Oligonucleotides	419
4.2. Polymer-Support Synthesis of Oligonucleotides	429
4.2.1. General Reaction Principle	430
4.2.2. Requirements for Supports and Reactants	430
4.2.3. Chemical Synthesis of Oligonucleotides on Supports	431
4.2.4. Enzymatic Synthesis of Oligo- and Polynucleotides on Supports	434
4.2.5. Miscellaneous Uses of Supports in Nucleotide Chemistry	455
4.3. Miscellaneous Methods in Chemical Oligonucleotide Synthesis	456
4.3.1. Chemical Synthesis via Activation of Hydroxyl Functions	457
4.3.2. Chemical Synthesis via Preactivated Phosphate Derivatives	457
4.3.3. Chemical Synthesis Using Unprotected Nucleotides	459
4.3.4. Chemical Synthesis on Complementary Templates	460
5. Formation of Internucleotide Linkages by Enzymic Reactions	461
5.1. Reactions Catalyzed by Polymerizing Enzymes	463
5.2. Reactions Catalyzed by Polynucleotide Ligases	464
5.3. Synthetic Reactions Catalyzed by Ribonucleases	471
References	478
	483

Acknowledgement. We are delighted to express our gratitude to many colleagues who supported our work by sending us reprints, preprints and/or unpublished results for incorporation into this review. We wish also to acknowledge several grants from the Deutsche Forschungsgemeinschaft for the support of our own work to which reference is made here.

Introduction

During the past decade solutions of an increasing variety of problems in the field of molecular genetics have rested on the availability of synthetic polynucleotides. Thus, to cite only a few examples, the elucidation of the genetic code was based on synthesis of the 64 possible trinucleoside diphosphates and on the preparation of polynucleotides containing repeating sequences (186, 249). More recently the development of synthetic procedures has culminated in the total synthesis of two tRNA-genes (2, 188, 190). A further useful application has been demonstrated in the use of synthetic oligomers of specific base sequence as specific primers for DNA sequencing (247, 366, 379, 467). Because of the many problems which remain with respect to our understanding in gene function or to future gene manipulation, it seems not

References, pp. 483-508

surprising that the effort for finding new methods or for improving earlier methods in polynucleotide synthesis still continues or even increases in many laboratories all over the world.

Accordingly a large number of contributions has appeared during the past three or five years which we will try to summarize in the present review. However, although we have attempted to give a broad survey of the entire field, comprehensiveness - deemed a hopeless task not only in the field of synthetic polynucleotides - could not be our major aim. We therefore offer our apologies to authors, whose contributions we could not recognize properly for reasons of space limitation or because their contributions are positioned more towards the periphery of the field. We have also limited ourselves to a compilation of those contributions made during the last 3-5 years, as the earlier literature is accessible through several excellent monographs (45, 184, 197, 258) and review articles (61, 65, 69, 185, 186, 319).

Abbreviations and Symbols

The system of abbreviations used in this review is principally that which has been suggested by the IUPAC-IUB commission in *J. Mol. Biol.* 55, 299 (1971). Thus, a monosubstituted terminal phosphoric acid residue is represented by a small p. Internal phosphoric diester 3'-5'-linkages are represented by a small p between the respective nucleoside symbols or by hyphens.

Nucleosides or nucleoside residues are represented by the following symbols: A adenosine, C cytidine, G guanosine, U uridine, T thymidine, I inosine, X xanthosine, Pu unspecified purine nucleoside, Py unspecified pyrimidine nucleoside, N or M unspecified nucleoside. The common 2'-deoxyribonucleosides are designated by the same symbols, modified in one of the following ways: small d is used as prefix preceding each residue or preceding whole chains, or small d is used as subscript at individual nucleoside symbols.

The diesterified phosphate residue, represented by a hyphen or by small p is considered to be attached to the oxygen atom of the 3'-carbon on its left and to that of the 5'-carbon on its right. For other types of linkage, the numerical form, as in 2'-5' or 5'-5' is used.

Examples of oligonucleotides: A-G-Up or ApGpUp represents a trinucleoside of the ribo series with internal 3'-5'-linkages and with a 3'-terminal phosphate. A-G-U>p represents the same trinucleotide but with terminal 2':3'-cyclic phosphate. pA-G-U is the same, but commencing with a 5'-phosphate and terminating in a uridine with unsubstituted 2'- and 3'-hydroxyls. d (pG-A-C-T) or dpG-A-C-T

is a tetranucleotide (all deoxy), with 5'-terminal phosphate on G. (rA)₆-(T₄-T₄) represents an octanucleotide with unsubstituted 5'- and 3'-terminal hydroxyls; the six 5'-terminal A-residues belong to the ribo series, whereas the two 3'-terminal T-residues belong to the deoxy series.

In polymerized nucleotides the prefix "poly" is usually substituted by the subscript *n* as in (dU)_{*n*}, which stands for poly dU. Non-covalent association between two polynucleotide chains, such as that ascribed to hydrogen-bonding, is indicated by a centre dot as in (rI)_{*n*} · (r2thioC)_{*n*}.

Symbols for N-protecting groups are: bz for benzoyl; an for anisoyl; ac for acetyl; ibu for isobutyryl. They are placed immediately before the single capital letters representing the nucleoside or nucleoside residue. In other cases they appear beginning with capital letters above the nucleoside symbols as in A^{bz}, dpG^{ibu}, or dpG^{ac}-C^{an}.

Symbols for O-protecting groups at the ribose or deoxyribose residues are: (MeOTr) or MMTTr for monomethoxytrityl, [(MeO)₂Tr] or DMTr for di-methoxytrityl, (Thp) or THP for tetrahydropyranyl, (Ac) or O-Ac for acetyl.

The condensing agents are commonly abbreviated by:

DCC for N,N'-dicyclohexyl-carbodiimide,

MS for mesitylene-sulfonyl-chloride,

TPS for 2,4,6-tri-isopropyl-benzene-sulfonyl-chloride.

Additional symbols for blocking groups etc. are indicated in the respective sections.

1. Protecting Groups

1.1. General Considerations

In natural polynucleotides the nucleotide monomers (Fig. 1.1) are exclusively linked by 3'-5'-phosphodiester linkages (Fig. 1.2). The formation of this linkage is normally the goal of the work done in chemical and enzymic oligo- and polynucleotide synthesis. In enzymic synthesis the specificity of the enzymes will only allow the "right" connection of the units. In a sequence-specific chemical synthesis several problems have to be solved in order to achieve a natural internucleotidic bond:

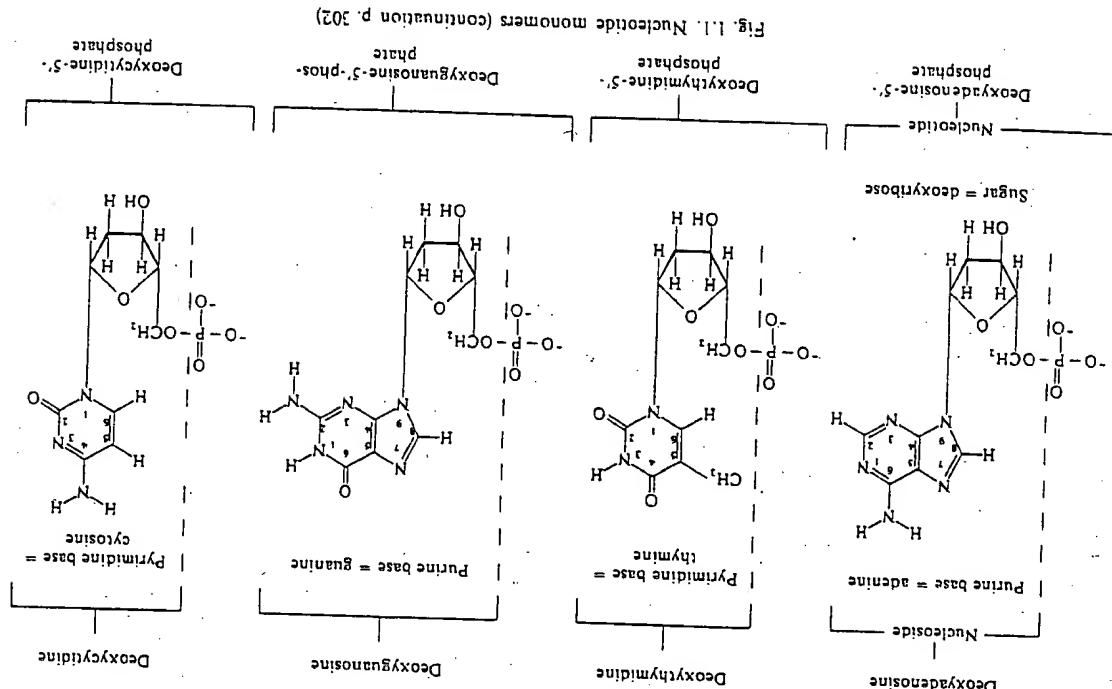
The intermediates have to be suitably protected.

Phosphorylation methods, suitable for the formation of internucleotidic bonds, have to be developed.

Techniques for the separation of reactants, products and by-products have to be elaborated.

References, pp. 483-508

The first three sections of this review describe recent advances toward the solution of these three problems.



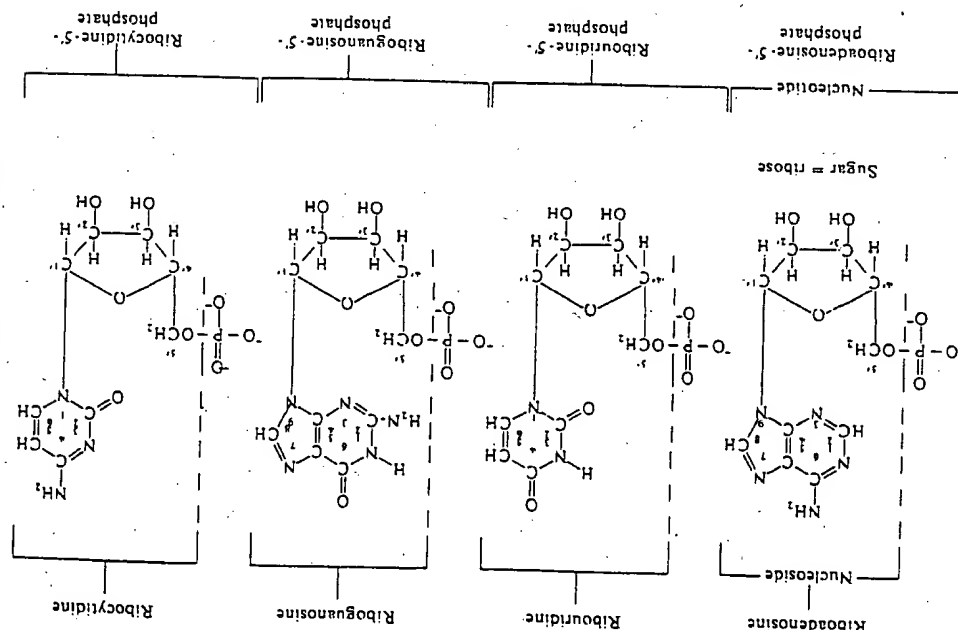


Fig. 1.1. Nucleotide monomers (continued from page 301).

the phosphate residue (with formation of pyrophosphates or branched-chain oligonucleotides). Other nucleophilic centers, e.g. N³ of pyrimidine bases, are generally not protected.

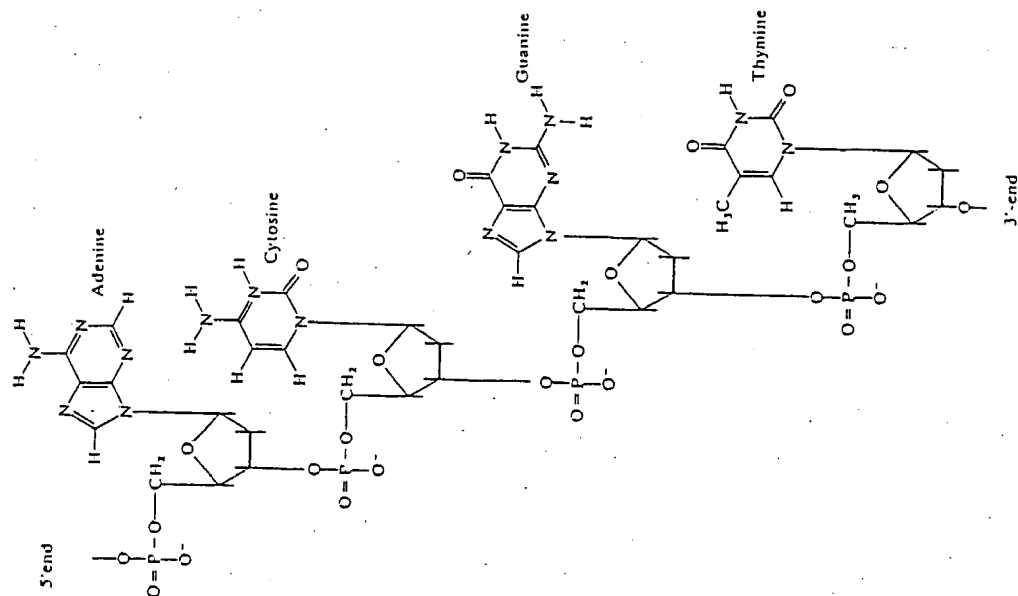


Fig. 1.2. Linkage of nucleotide monomers

The necessity for protection of different functions of the nucleotide molecule arises from the fact that several nucleophilic centers (see Fig. 1.1) are able to react with an activated nucleotide. These are: the 3'- and 5'-hydroxyl groups, additionally the 2'-hydroxyl group in ribonucleic acid constituents, the amino groups of the nucleobases,

References, pp. 483-508

The blocking groups used for the different functions of the nucleoside or nucleotide molecule and the conditions for their introduction and removal will be discussed. Earlier work in this field (322) has been reviewed in several articles and monographs cited in the introduction. Recently, an excellent treatment of the protecting groups for phosphoric acids, incl. nucleotides, has been given by F. ECKSTEIN (32).

1.2. Choice of Blocking and Deblocking Conditions

The following considerations, in principle, govern the choice of reagents for blocking and deblocking of oligo- and polynucleotides and their constituents:

1. The protecting groups must be stable during the formation of an internucleotide linkage. At a later stage of the oligonucleotide synthesis it must be possible to remove them without alteration of the original function.
2. Protection and deprotection must proceed without rupture or isomerization of previously formed internucleotide bonds.
3. The same is required for "weak spots" of the nucleotide molecule itself, especially the glycosidic linkage.
4. Introduction and removal of blocking groups should be, as far as possible independent of reactions of other blocking groups of the same molecule, and *vice versa*.
5. Steric and electronic effects of blocking groups should not be adverse to the formation of internucleotidic linkages.

A closer look at the considerable number of protecting groups described for use in nucleic acid chemistry reveals that only very few will meet all these requirements (see Table 1.1 and Section 1.3 for a detailed discussion). Even the basic criteria 1 and 2 are not met in all cases. Thus, for example, the strongly alkaline removal of N-acyl groups may be accompanied by deamination of the cytosine base, and the use of benzyl groups for protection of the internucleotidic linkage was abandoned due to partial scission of the latter on anionic debenzoylation. The use of such groups or conditions may, nevertheless, be necessary as a compromise. Criteria 2 and 3 also exclude a number of groups from use in the deoxy resp. ribo series due to the strongly acid resp. alkaline conditions necessary for their removal

References, pp. 483—503

(see Sections 1.3 and 1.6). This is because the glycosidic linkage of N-substituted purine-deoxyribonucleotides is very sensitive to media of pH < 4, and the ribo-internucleotide bond is easily cleaved at pH > 10 due to the neighbouring effect of the 2'-OH. Criterion 4 is of importance in the design of specifically blocked intermediates for stepwise oligonucleotide synthesis. As discussed in Sections 1.5 and 1.6, the number of groups, which fulfil all requirements of selectivity is very small, and various strategies for the selective blocking or deblocking of certain functions have been developed to overcome this difficulty. Criterion 5 is hard to take into account, since little is known for instance about conformations of blocked nucleotides in non-aqueous media and steric effects of blocking groups on intermediate states of the phosphorylation reaction. Such effects have been described, for example for the "shielding" of 3'-hydroxyl groups of ribonucleosides by blocking groups at the 2'-function and *vice versa* (see Section 1.6).

Very recently attention has been drawn to the use of blocking groups in stepwise enzymatic oligonucleotide synthesis. This new approach, realized with the enzyme polynucleotide phosphorylase, is described in Section 5. The blocking groups used in this case have been selected by testing a great number of candidates. Selection of the most suitable protecting agent for this purpose would be greatly simplified by a more detailed knowledge of the steric and electronic environment of the active site of the enzymes in question.

1.3. Survey of Blocking Groups

A list of blocking groups for use in nucleotide chemistry is given in Table 1.1. Of course, it is impossible to take into account every group tested for blocking purposes in laboratories all over the world or to go into detail on all the different conditions elaborated for introduction and removal of certain groups. However, the information given in Table 1.1 is not restricted to the most commonly used groups, but attempts to draw attention also to other groups which have been described, but perhaps not fully exploited as to their potentiality. This is certainly justified by the fact that any discrimination between "useful" and "unuseful" blocking groups would be arbitrary. Moreover, protecting groups rejected for the synthesis of one intermediate may well serve for the preparation of another. The discussion in Sections 1.5 and 1.6 will demonstrate how such intermediates for different approaches to oligonucleotide synthesis are built up by combination of different groups.

Table 1.1 is subdivided in the following way:

In column 1 the protecting groups are numbered according to structural similarity and degree of substitution. These numbers will be referred to in the further discussion. In column 2 the blocking groups are named and classified according to structural similarity. Column 3 lists standard abbreviations. These are a) recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature, for amino blocking groups, resp. b) for terminal radicals; c) abbreviations as used in publications of H. G. KHORANA and coworkers, if different from a) and b); d) abbreviations as used by other authors cited as references, if different from a) and b). Column 4 gives representations of the structural formulae. In column 5 the functions are listed, for which the resp. blocking groups can be used. It is convenient to distinguish between the following functions to be blocked: phosphomonoester resp. phosphodiester residues (i.e. terminal phosphate or internucleotidic bonds), hydroxyl- and amino groups in general (including the 2'- and/or 3'-hydroxyl groups of ribosides, if separately blocked), the vicinal diol group of ribosides in cases, in which it reacts as one unit. Cases, in which the blocking group is introduced selectively into one out of several similar functions, are indicated by e.g. "selectively 5'-OH" etc. Column 6 gives the appropriate reagents for blocking. More detailed reaction conditions are listed only if necessary for reasons of selectivity. Similarly in columns 7 and 8 the reagents for deblocking and the groups which are deblocked under these conditions are listed. The latter are identical with the blocked moieties except for cases of selective deblocking, which, thus, can be easily discerned. Column 9 points to special applications of blocking groups. The following cases are listed: a) blocking groups which allow solvent extraction of oligonucleotides, b) blocking groups rendering possible the separation of oligonucleotides by affinity chromatography, c) activable blocking groups, d) blocking groups for enzymatic monoaddition substrates. A detailed discussion follows in Section 1.4.

Column 10 of Table 1.1 contains all literature references pertinent to the different blocking groups. Since this column provides ample literature information, we will, in the following sections, limit ourselves to citing only those publications, which may serve to illustrate those points which are especially stressed and discussed. It should be said in conclusion, that this survey does not include all those groups and reagents, which are used for other than blocking purposes, e.g. groups used for selective base modification in tRNA or other polynucleotides, even if they would, in principle, meet some of the requirements for protecting groups listed in Section 1.2.

References, pp. 483-508

Table 1.1. Blocking Groups in Nucleotide and Polynucleotide Chemistry*

No. Blocking group	Abbrev-Structural formula	Blocked moiety	Conditions for blocking	Deblocked moiety	Special applic-conditions	References
1 β-substituted ethyl esters: β-cyanoethyl- CNEt ⁺	$\text{--O--CH}_2\text{--CH}_2\text{--CN}$	phospho- mono- β-cyano- nucleoside, phospho- mono- alkali	ester, ethyl-phos- phate, DCC	nucleotide, hydrazide, nitrile, DCC	(6, 42, 74, 113, 189, 197, 235, 417, 418, 419)	
2 2-cyano-1-methyl-ethyl	$\text{--O--CH(CH}_3\text{)--CH}_2\text{--CN}$	phospho- nucleotide, phospho- mild	ester, mono- alkali	phospho- mono- alkali	(65)	
3 2-acetyl-2-methyl-ethyl	$\text{--O--CH(CH}_3\text{)--CH(CH}_3\text{)--C(=O)CH}_3$	phospho- nucleotide, phospho- alkali	ester, mono- alkali	phospho- mono- alkali	(65)	
4 2-acetyl-1-methyl-ethyl	$\text{--O--CH(CH}_3\text{)--CH}_2\text{--C(=O)CH}_3$	phospho- nucleotide, phospho- alkali	ester, mono- alkali	phospho- mono- alkali	(65)	

* For explanations see text of p. 1.3 (p. 305).

Table 1.1 (continued)

No.	Blocking group	Abbreviation	Structural formula	Blocked moiety	Conditions for blocking	Deblocked moiety	Deblocking conditions	Special applications	References
5	2-sulfolien-4-yl			phospho- mono- ester	nucleotide, 4-hydroxy- 2-sulfolene, DCC	phospho- mono- ester	alkali		(65)
6	2(α-pyridyl)- ethyl			phospho- mono- ester	nucleotide, α-pyridyl- ethanol, DCC	phospho- mono- ester	NaOCH ₃ in methanol/ pyridine		(97, 98)
7	2-(phenyl- carbonyl)- ethyl			phospho- mono- ester	nucleotide, phenyl- hydra- crylamide, DCC	phospho- mono- ester	alkali	affinity	(7, 289, 290)
8	2-(p-methoxy- phenylcarba- moyl-)ethyl	MPH ^d		phospho- mono- ester	nucleotide, p-methoxy- phenyl- hydracryl- amide, DCC	phospho- mono- ester	alkali	affinity	(289, 290)
9	2-(benzyl- carbamoyl)- ethyl			phospho- mono- ester	nucleotide, benzylhydra- crylamide, DCC	phospho- mono- ester	alkali	affinity	(289, 290)
10	2-(phenyl- mercapto-) ethyl	PME ^d		phospho- mono- ester	nucleotide, 2-phenyl- mercapto- ethanol, DCC	phospho- mono- ester	1) periodate 2) alkali	affinity extrac- tion	(7, 290a, 422, 465)
11	9-fluorenyl- methyl			phospho- mono- ester	nucleotide, 9-fluorenyl- methanol, TPS	phospho- mono- ester	alkali	extrac- tion	(176)
12	2',3'-(2,4- dimethoxy- benzylidene-) uridyl			phospho- mono- ester	nucleotide, 2',3'-(2,4- dimethoxy- benzylidene-) uridine, DCC	phospho- mono- ester	1. mild acid 2. NaJO ₄ 3. alkali		(177, 178, 398a)
13	β,β-tri- chloroethyl	Cl ₃ Et ^d		phospho- mono- ester, phospho- diester	nucleotide, β,β-tri- chloro- ethanol, DCC	phospho- mono- ester, phospho- diester	Zn/Cu in DMF		(42, 75, 76, 78, 94, 197, 296, 298)

Table I.1 (continued)

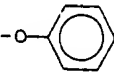
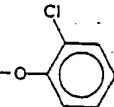
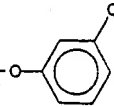
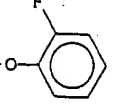
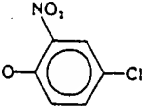
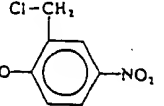
No.	Blocking group	Abbrev- iation	Structural formula	Blocked moiety	Conditions for blocking	Deblocked moiety	Deblocking conditions	Special applications	References
					nucleoside, β, β, β -tri-chloroethyl-phosphate TPS nucleoside, β, β, β -tri-chloroethyl-phosphorodichloridate nucleoside, β, β, β -tri-chloroethyl- β -cyano-ethyl-phosphochloridate				
<i>misc. ester groups:</i>									
14	phenyl-			phosphodiester	nucleoside, phenyl-phosphodichloridate nucleoside, phenyl-phosphate, TPS	phosphodiester	strong alkali		(31, 73 350, 352)
15	o-chlorophenyl-			phosphodiester	nucleoside, o-chlorophenyl-phosphate, TPS	phosphodiester	alkali		(352)
16	m-chlorophenyl-			phosphodiester	nucleoside, m-chlorophenyl-phosphate, TPS	phosphodiester	alkali		(352)
17	o-fluorophenyl-			phosphodiester	nucleoside, o-fluorophenyl-phosphate, TPS	phosphodiester	alkali		(352)
18	4-chloro-2-nitro-phenyl-			phosphomono-ester	nucleotide, 4-chloro-2-nitro-phenol, DCC	phosphomono-ester	strong alkali		(289)
19	4-nitro-2-chloro-methyl-phenyl-			phosphomono-ester	nucleoside, 4-nitro-2-chloro-methyl-phenyl-phosphate, DCC	phosphomono-ester	aqueous pyridine	activation	(281)

Table 1.1 (continued)

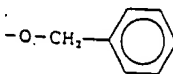
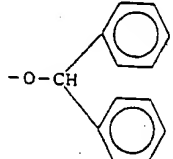
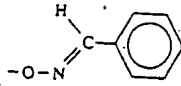
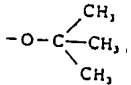
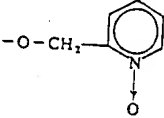
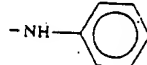
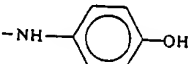
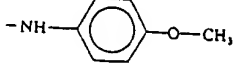
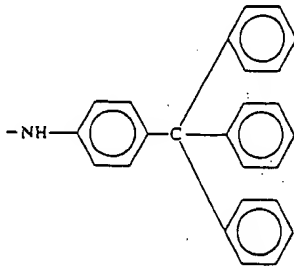
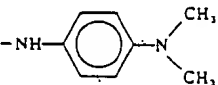

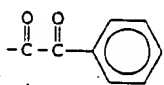

No.	Blocking group	Abbreviation	Structural formula	Blocked moiety	Conditions for blocking	Deblocked moiety	Deblocking conditions	Special applications	References
20	benzyl-	Bzl ^b		phospho- mono- ester	nucleoside, benzyl- phospho- dichloridate	phospho- mono- ester	Pd/H ₂		(197)
				phospho- diester	nucleotide, phenyl- diazo- methane	phospho- diester	NaI in acetonitrile		(369)
21	benzhydryl-			phospho- mono- ester	nucleotide, diphenyl- diazo- methane	phospho- mono- ester	acid		(63, 64)
22	benzaloxime ester			phospho- mono- ester	benzal- doxime + nucleoside- 5'-phosphor- morpho- lidate	phospho- mono- ester	alkali	affinity	(290)
23	ethylthio-	EtS ^a	$-S-CH_2-CH_3$	phospho- mono- ester	S-ethyl- phosphoro- thioate, nucleoside, DCC	phospho- mono- ester	I ₂ / pyridine	activ- ation	(55, 56, 58, 129, 146, 488)
24	<i>t</i> -butyl-			phospho- mono- ester	<i>t</i> -butanol, DCC, nucleotide	phospho- mono- ester	acid		(63, 489)
25	1-oxido- 2-picolyl-			phospho- mono- ester	nucleotide, 1-oxido- pyridine- 2-yl-diazo- methane	phospho- mono- ester	1) acetic anhydride 2) methanol, ammonia		(83, 271)
phosphoramidate groups:									
26	anilidate	PhNH		phospho- mono- ester	nucleotide, aniline, DCC	phospho- mono- ester	isoamyl- nitrite, pyridine/ acetic acid		(309, 310, 312)
27	<i>p</i> -hydroxy- anilidate			phospho- mono- ester	nucleotide, <i>p</i> -hydroxy- aniline, DCC	phospho- mono- ester	isoamyl- nitrite, pyridine/ acetic acid	activ- ation	(316)
28	<i>p</i> -methoxy- anilidate			phospho- mono- ester	nucleotide, <i>p</i> -methoxy- aniline, DCC	phospho- mono- ester	isoamyl- nitrite, pyridine/ acetic acid		(309)

Table 1.1 (continued)

No.	Blocking group	Abbreviation	Structural formula	Blocked moiety	Conditions for blocking	Deblocked moiety	Deblocking conditions	Special applications	References
29	<i>p</i> -(trityl-)anilidate	TPM ^c		phospho- mono- ester	nucleotide, <i>p</i> -amino- phenyl- triphenyl- methane, DCC	phospho- mono- ester	isoamyl- nitrite, pyridine, acetic acid	affinity extraction	(3)
30	<i>p</i> -(<i>N,N</i> -dimethylamino-)anilidate			phospho- mono- ester	nucleotide, <i>N,N</i> -di- methyl- <i>p</i> - phenylene diamine, DCC ^c	phospho- mono- ester	isoamyl- nitrite, pyridine, acetic acid	affinity	(136, 138, 435)
<i>ester groups:</i>									
31	formyl-			OH	formic anhydride formic acid <i>N</i> -formyl- imidazole	OH	mild alkali, aqueous pyridine		(63, 99, 332, 398, 418, 419, 477)
				sel. 2'/ 3'-OH	trimethyl- orthoformate <i>p</i> -toluene- sulfonic acid (via methoxy- methylidene)				(117)
						sel. 5'-OH	methanol		(477)
32	benzoyl-formyl			-OH	benzoyl- formyl- chloride	-OH	aqueous pyridine		(234)
33	acetyl-	ac ^a Ac ^b		-OH, -NH ₂	acetic anhydride	-OH -NH ₂	alkali, ammonia		(184, 197, 258)
				sel. -OH	acetic anhydride, H ₂ O				(43, 429a)
				sel. -OH	acetic anhydride, BF ₃ -ether				(346)

H. KÖSEL and H. SELIGER:

Recent Advances in Polynucleotide Synthesis

315

Table 1.1 (continued)

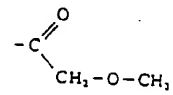
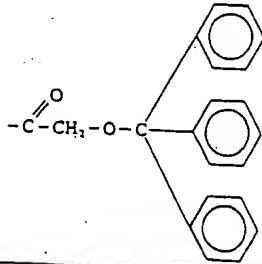
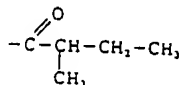
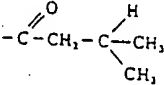
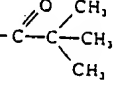
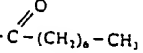

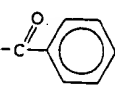
No.	Blocking group	Abbrev. iation	Structural formula	Blocked moiety	Conditions for blocking	Deblocked moiety	Deblocking conditions	Special applications	References
33	acetyl (continued)			-OH	dioxane, acetonitrile, HCl				(482)
				sel. NH ₂	acetic anhydride, DMF, tri-n-butylamine				(329, 330)
				sel. 2'/3'-OH	trimethyl-orthoacetate (via methoxy-ethylidene-)				(99)
				sel. 3'-OH	8-hydroxy-quinoline N-acetate				(268)
				sel. -NH ₂	5-(acetyl-oxymino)-2,6-dioxo-4-(methyl-imino)-1,3-dimethyl-hexahydro-pyrimidine				(28)
				sel. 5'-OH	acetic anhydride, diethylazodicarboxylate, tri-phenylphosphine				(264)
				sel. α-NH ₂ of amino-acyl-nucleoside	5-chloro-8-hydroxy-quinoline-O-acetate				(47)
						sel. -OH	strong alkali		(184, 197, 258)
34	methoxy-acetyl			-OH	methoxy-acetic anhydride, trimethyl-methoxy-orthoacetate	-OH	alkali		(351, 352, 353)
35	triphenyl-methoxy-acetyl	trac ^d		-OH	triphenyl-methoxy-acetic acid, triisopropyl-benzene-sulfonyl-chloride	-OH	mild alkali	affinity	(463)

Table 1.1 (continued)

No.	Blocking group	Abbreviation	Structural formula	Blocked moiety	Conditions for blocking	Deblocked moiety	Deblocking conditions	Special applications	References
36	phenoxyacetyl			-OH	phenoxy acetic anhydride	-OH	alkali		(351)
37	<i>p</i> -chloro-phenoxy-acetyl			-OH	<i>p</i> -chloro-phenoxy-acetic anhydride	-OH	alkali		(353)
38	chloroacetyl			-OH	chloroacetic anhydride	-OH	alkali; 2-mercapto-ethylamine, neutral		(57)
39	trichloro-acetyl			-OH	trichloro-acetic anhydride	-OH	alkali		(197)
40	diphenyl-chloroacetyl			-OH	diphenyl-chloro-acetyl-chloride	-OH	alkali; thiourea, neutral		(57)
41	trifluoro-acetyl	F ₃ CCO ^b		-OH	trifluoro-acetic anhydride	-OH	alkali		(197)
42	propionyl			-OH, -NH ₂	propionic anhydride	-OH, -NH ₂	alkali, ammonia		(159, 160)
43	dihydrocinnamoyl			-OH	dihydro-cinnamoyl chloride (anhydride)	-OH	chymo- trypsin in acetonitrile/ phosphate buffer pH 7		(363, 437)
44	β -benzoyl-propionyl-	βB^d		-OH	β -benzoyl-propionic acid DCC	-OH	hydrazine in pyridi- mium acetate		(112, 230, 470)
45	<i>n</i> -butyryl			-OH, -NH ₂	butyric anhydride	-OH, -NH ₂	alkali, ammonia		(159, 160)
46	isobutyryl-	iB ^e iBu ^e		-OH, -NH ₂	isobutyric anhydride	-OH, -NH ₂ sel. -OH	alkali, ammonia strong alkali		(37, 457)

Table 1.1 (continued)

No.	Blocking group	Abbreviation	Structural formula	Blocked moiety	Conditions for blocking	Deblocked moiety	Deblocking conditions	Special applications	References
47	2-methylbutyryl	mB ^a		-OH, -NH ₂	2-methylbutyric anhydride	-OH, -NH ₂ sel. -OH	alkali, ammonia strong alkali		(41)
48	isovaleryl			-OH	isovaleryl chloride	-OH	alkali	mono-addition substrate	(179, 180)
49	pivaloyl-(trimethylacetyl)			-OH, -NH ₂	pivaloyl chloride	-OH, -NH ₂	alkali, ammonia		(99)
50	octanoyl-			-OH, -NH ₂	octanoic anhydride	-OH, -NH ₂	alkali, ammonia		(159, 160)
51	linoleyl-			-OH, -NH ₂	linoleic anhydride	-OH, -NH ₂	alkali, ammonia		(159, 160)
52	benzoyl-	bz ^a Bz ^b		-OH, -NH ₂	benzoyl chloride	-OH, -NH ₂ sel. -OH	alkali, ammonia n-butylamine for G ^{bz} strong alkali		(184, 197, 258, 315, 457)
				sel. -NH ₂	benzoic acid-N-hydroxy-succinimide ester				(303)
				sel. 5'-OH	benzoic acid, diethylazodicarboxylate, triphenylphosphine				(264)
						sel. -NH ₂	hydrazine in pyridine-acetate		(233)
				-OH, -NH ₂	benzoyl cyanide				(157)
				sel. -OH	benzoic acid anhydride/H ₂ O				(43)

H. Kössel and H. Seliger:

Recent Advances in Polynucleotide Synthesis

Table 1.1 (continued)

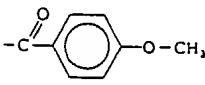
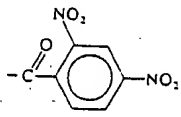
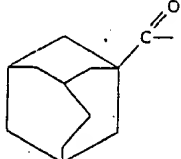
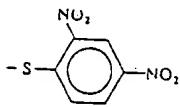

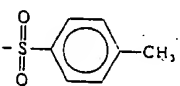

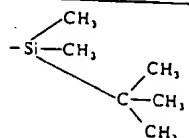
No.	Blocking group	Abbreviation	Structural formula	Blocked moiety	Conditions for blocking	Deblocked moiety	Deblocking conditions	Special applications	References
52	benzoyl (continued)			sel. 2'(3')-OH	trimethyl-orthobenzoate, p-toluenesulfonic acid (via methoxy benzylidene)				(99)
53	anisoyl	an ^a An ^b		-OH, -NH ₂	anisoyl chloride	-OH, -NH ₂ sel. -OH	alkali, ammonia strong alkali		(197)
54	dinitrobenzoyl-			-OH, -NH ₂	dinitrobenzoyl chloride	-OH, -NH ₂	alkali, ammonia		(197)
55	adamantoyl-			-OH	adamantane-carbonyl chloride	-OH	alkali		(253, 405)
56	dinitrobenzenesulfonyl-			-OH	2,4-dinitrobenzenesulfonyl chloride	-OH	thiophenol, neutral		(112, 197)
57	mesyl-			-OH, -NH ₂	methanesulfonyl chloride	-OH, -NH ₂	alkali		(197)
58	tosyl	Tos ^b		-OH	p-toluene-sulfonyl chloride	-OH	alkali		(10, 197, 162a, 162b, 162c)
59	trimethylsilyl	TMS ^b		-OH, -NH ₂	trimethylchlorosilane, bis-trimethyltrifluoroacetamide	-OH, -NH ₂	weak acid or weak alkali		(197)
60	t-butyl-dimethylsilyl			-OH	t-butyl-dimethylsilyl chloride	-OH	NR ₄ ⁺ F ⁻ neutral		(307)

Table 1.1 (continued)

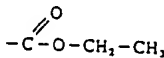
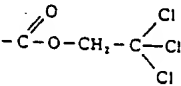
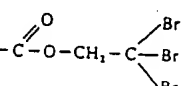
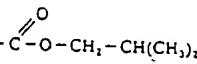
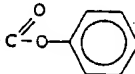
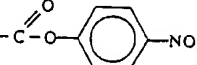
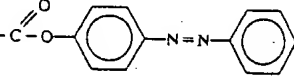
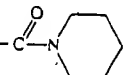
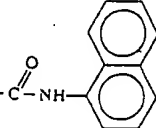
No.	Blocking group	Abbreviation	Structural formula	Blocked moiety	Conditions for blocking	Deblocked moiety	Deblocking conditions	Special applications	References
61	ethyloxy-carbonyl-			-OH	nucleoside chloroformate + ethanol	-OH	alkali		(391)
62	trichloro-ethyl-oxycarbonyl			-OH, -NH ₂	trichloro-ethylchloroformate	-OH, -NH ₂	Zn/acetic acid (methanol)		(183, 466)
63	tribromoethyl-oxycarbonyl-			-OH	tribromo-ethylchloroformate	-OH	Zn/Cu in acetic acid		(54)
64	isobutyl-oxycarbonyl-	BOC ^a		-OH, -NH ₂ , sel. 5'-OH	isobutyl-chloroformate	-OH, -NH ₂ , sel. -OH	alkali ammonia strong alkali		(236, 304)
65	phenyloxy-carbonyl-			-OH	phenyl-chloroformate	-OH	alkali		(14)
66	p-nitrophenyl-oxycarbonyl-			-OH	p-nitro-phenyl-chloroformate; nucleoside chloroformate + p-nitrophenol	-OH	alkali, ammonia		(229, 391)
67	p-phenylazo-phenyloxy-carbonyl-			-OH, sel. 5'-OH	nucleoside chloroformate + p-phenylazo-phenol	-OH	alkali	affinity	(391)
68	piperidine-carbamoyl-			-OH	nucleoside chloroformate + piperidine	-OH	alkali		(391)
69	naphthyl-carbamoyl-			-OH	naphthyliso-cyanate	-OH	alkali	affinity	(4)

Table 1.1 (continued)

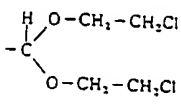
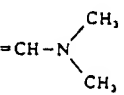
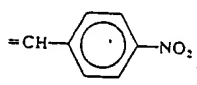
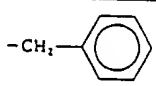
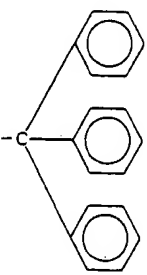
No. Blocking group	Abbrev. Structural formula iation	Blocked moiety	Conditions for blocking	Deblocked moiety	Deblocking conditions	Special applications	References
<i>Schiff bases, orthoesters and deriv.</i>							
71 bis-(2-chloroethyl)-orthoformate		-OH	2-chloroethylorthoformate	-OH	acid		(133)
72 N,N-dimethyl-amino-methylidene		-NH ₂ selective	dimethyl-formamide-acetals	-NH ₂	alkali, mild acid		(153, 154, 155, 197, 397, 398, 420)
73 p-nitro-benzylidene		-NH ₂ (concurrent with No. 123)	1. nitro-benzaldehyde, HC(OEt) ₃ , F ₃ C-COOH, DMF 2. benzoyl-chloride	-NH ₂	mild acid		(484)
<i>ether groups:</i>							
74 benzyl-	bzl ^a Bzl ^b 	-OH, -NH ₂	benzyl-chloride/ alkali	-OH, -NH ₂	H ₂ /Pd		(197)
		-OH, -NH ₂ , sel. 2'-OH	benzyl-chloride/ NaH				(25, 26, 27, 163, 192)
		-OH, -NH ₂	phenyldiazo-methane, SnCl ₂				(52)
		sel. -NH ₂	nucleoside-Na ⁺ -salt + benzyl-chloride				(390)
75 triphenyl-methyl-	Tr ^a Tr ^b 	-OH, -NH ₂ selective 5'-OH	triphenyl-methyl-chloride	-OH, -NH ₂	acid		(184, 197, 354)
				-OH, -NH ₂	silicagel		(225)
				sel. 2'-OH	acid		(197)
				sel. 3'-OH	acid		(197)
				sel. 5'-OH	acid		(88, 218a, 354)
					affinity		(41)

Table I.1 (continued)

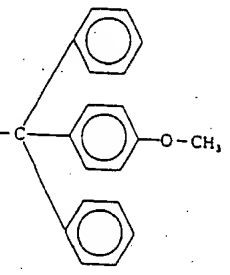
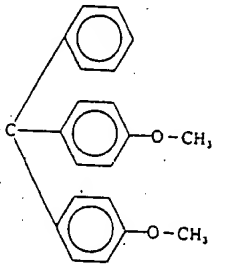
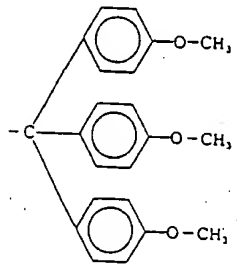
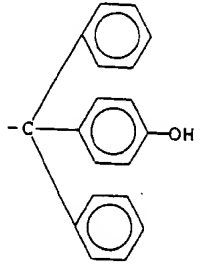
No. Blocking group	Abbreviation	Structural formula	Blocked moiety	Conditions for blocking	Deblocked moiety	Deblocking conditions	Special applications	References
76 <i>p</i> -methoxy-triphenyl-methyl	mmt ^a MeOTr ^b MMTr ^c		-OH, -NH ₂ selective 5'-OH	<i>p</i> -methoxy-triphenyl-methyl-chloride	-OH, -NH ₂	mild acid	affinity	(184, 197) (41, 398b)
77 <i>p,p'</i> -dimethoxy-triphenyl-methyl	dmt ^a (MeO) ₂ Tr ^b DMTr ^c		-OH, -NH ₂ selective 5'-OH	<i>p,p'</i> -dimethoxy-triphenyl-methyl-chloride	-OH, -NH ₂	very mild acid	affinity	(41, 197)
78 <i>p,p',p''</i> -trimethoxy-triphenyl-methyl			-OH, -NH ₂ selective 5'-OH	<i>p,p',p''</i> -trimethoxy-triphenyl-methyl-chloride	-OH, -NH ₂	extremely mild acid		(197)
79 <i>p</i> -hydroxy-trityl	pHOTr ^d		OH-sel. 5'-OH	<i>p</i> -hydroxy-phenyl-diphenyl-methyl-chloride	OH-	mild acid		(436)

Table 1.1 (continued)

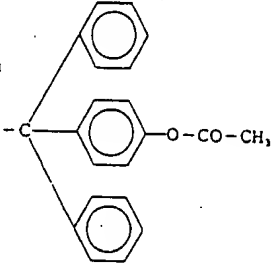
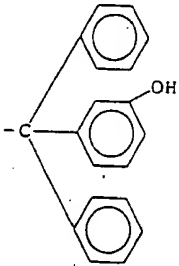
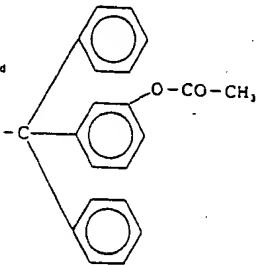
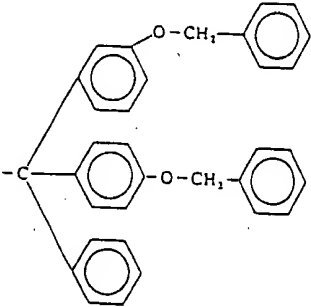
No.	Blocking group	Abbreviation	Structural formula	Blocked moiety	Conditions for blocking	Deblocked moiety	Deblocking conditions	Special applications	References
80	<i>p</i> -acetoxy-trityl	pAcOTr ^d		OH-sel. 5'-OH	<i>p</i> -acetoxy-phenyl-diphenyl-methyl-chloride	OH-	mild acid		(436)
81	<i>m</i> -hydroxy-trityl	mHOTr ^d		OH-sel. 5'-OH	<i>m</i> -hydroxy-phenyl-diphenyl-methyl-chloride	OH-	mild acid		(436)
82	<i>m</i> -acetoxy-trityl	mAcOTr ^d		OH-sel. 5'-OH	<i>m</i> -acetoxy-phenyl-diphenyl-methyl-chloride	OH-	mild acid		(436)
83	di-(<i>p</i> -benzyloxy-)trityl	DPTTr ^d		OH-sel. 5'-OH	di(benzyloxyphenyl)-phenyl-methyl-chloride	OH-	very mild acid		(436)

Table 1.1 (continued)

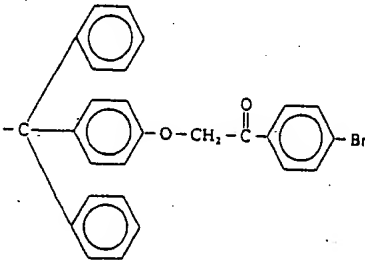
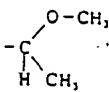
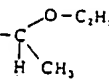
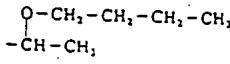
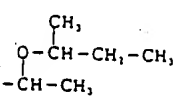
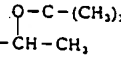
No. Blocking group	Abbreviation	Structural formula	Blocked moiety	Conditions for blocking	Deblocked moiety	Deblocking conditions	Special applications	References
84	bromo-phenacyl-trityl-	BPTTr ^d		-OH selective 5'-OH	p-bromo-phenacyloxy-phenyl-diphenyl-methyl-chlorid	-OH very mild acid		(436)
<i>acetal, ketal groups</i>								
85	α-(methoxy-ethyl)		-OH	methylvinyl-ether p-toluene-sulfonic acid acetaldehyde + methanol in DMF	-OH	mild acid	mono addition substrate	(252) (386a, 387)
86	α-ethoxy-ethyl-	EtOEt ^b EE ^d 	-OH, -NH ₂	ethylvinyl ether	-OH, -NH ₂	mild acid		(197)
87	n-butoxy-ethyl		-OH,	n-butylvinyl-ether, trifluoroacetic acid acetaldehyde + n-butanol in DMF	-OH	mild acid		(386a, 387)
88	sec-butoxy-ethyl		-OH	sec-butylvinyl ether, trifluoroacetic acid acetaldehyde + sec-butanol in DMF	-OH	mild acid		(386a, 387)
89	t-butoxy-ethyl		-OH	tert-butyl vinyl ether, trifluoroacetic acid	-OH	mild acid		(386a, 387)

Table 1.1 (continued)

No. Blocking group	Abbrev. Structural formula	Blocked moiety	Conditions for blocking	Deblocked moiety	Deblocking conditions	Special applications	References
90 α -methoxyisopropyl-	$\begin{array}{c} \text{OCH}_3 \\ \\ -\text{C}-\text{CH}_3 \\ \\ \text{CH}_3 \end{array}$	-OH sel. 5'-OH	2-methoxypropylene acid 2,2-dimethoxypropane, diphenylphosphate, dimethylacetamide	-OH	mild acid		(197)
91 isopropoxyisobutyl-	$\begin{array}{c} \text{O}-\text{CH}(\text{CH}_3)_2 \\ \\ -\text{CH}-\text{CH}(\text{CH}_3)_2 \end{array}$	-OH	isopropoxyisobutylene trifluoroacetic acid; isobutyric aldehyde + isopropanol in DMF	-OH	mild acid		(386a, 387)
92 <i>n</i> -butoxyisobutyl	$\begin{array}{c} \text{O}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_3 \\ \\ -\text{CH}-\text{CH}(\text{CH}_3)_2 \end{array}$	-OH	isobutyric aldehyde + <i>n</i> -butanol in DMF <i>n</i> -butoxyisobutylene trifluoroacetic acid	-OH	mild acid		(386a, 387)
93 isobutoxyisobutyl-	$\begin{array}{c} \text{O}-\text{CH}_2-\text{CH}(\text{CH}_3)_2 \\ \\ -\text{CH}-\text{CH}(\text{CH}_3)_2 \end{array}$	-OH	isobutoxyisobutylene trifluoroacetic acid isobutyric aldehyde + isobutanol in DMF	-OH	mild acid		(386a, 387)
94 2-methoxyethoxyisobutyl	$\begin{array}{c} \text{O}-\text{CH}_2-\text{CH}_2-\text{O}-\text{CH}_3 \\ \\ -\text{CH}-\text{CH}(\text{CH}_3)_2 \end{array}$	-OH	isobutyraldehyde + 2-methoxyethanol, trifluoroacetic acid	-OH	mild acid		(387)
95 1-methoxycyclohexyl-	$\begin{array}{c} \text{H}_3\text{C}-\text{O} \\ \\ \text{Cyclohexyl} \end{array}$	-OH	1-methoxycyclohexene, acid	-OH	mild acid		(197)

Table 1.1 (continued)


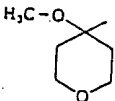
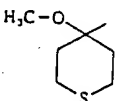
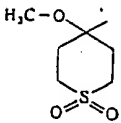
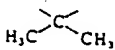
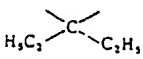
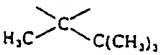
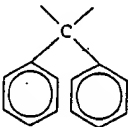
No.	Blocking group	Abbreviation	Structural formula	Blocked moiety	Conditions for blocking	Deblocked moiety	Deblocking conditions	Special applications	References
96	tetrahydropyranyl-	thp ^a Thp ^b THP ^c		-OH, -NH ₂	dihydropyran p-toluene-sulfonic acid		acid		(184, 197, 258)
97	methoxy-tetrahydropyranyl-			-OH	4-methoxy-dihydropyran, p-toluene-sulfonic acid	sel. -OH	mild acid		(297, 299)
98	methoxy-tetrahydrothiopyranyl			-OH	4-methoxy-5,6-dihydro-4H-thiopyran, m-silylene sulfonic acid	-OH	mild acid		(32)
99	corresp. sulfone of No. 98			-OH	methoxy-tetrahydrothio-pyranyl-nucleoside + m-chloro-perbenzoic acid	-OH	acid		(32)
<hr/>									
	acetal, ketal groups				Sugar vicinal diol				
100	isopropylidene-	>CMe ₂ ^a		2',3'-(-OH) ₂	acetone, HCl (2',3'-OH) ₂ 2,2-di-methoxy-propane, p-toluene-sulfonic acid		strong acid		(99, 100, 101, 184, 197, 258)
101	diethylmethylenidene			2',3'-(-OH) ₂	diethyl-ketone, HCl	2',3'-(-OH) ₂	strong acid		(197)
102	methyl-t-butyl-methylenidene-			2',3'-(-OH) ₂	methyl-t-butylketone, HCl	2',3'-(-OH) ₂	strong acid		(197)
103	diphenyl-methylenidene-			2',3'-(-OH) ₂	diphenyl-ketone, HCl	2',3'-(-OH) ₂	strong acid		(197)

Table 1.1 (continued)

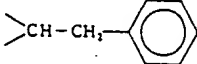
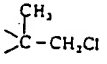
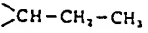
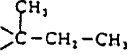
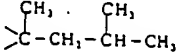
No.	Blocking group	Abbreviation	Structural formula	Blocked moiety	Conditions for blocking	Deblocked moiety	Deblocking conditions	Special applications	References
104	2-phenylethylidene-			2',3'- (-OH) ₂	2-phenyl-acetaldehyde, (di)ethylphosphorothioate, 2,2-dimethoxypropane, dimethylformamide	2',3'- (-OH) ₂	strong acid		(100, 101)
105	2-chloro-1-methylethylidene			2',3'- (-OH) ₂	methyl chloromethylketone, (di)ethylphosphorothioate, 2,2-dimethoxypropane, dimethylformamide	2',3'- (-OH) ₂	acid		(100, 101)
106	n-propylidene-			2',3'- (-OH) ₂	propionaldehyde, (di)ethylphosphorothioate, 2,2-dimethoxypropane, dimethylformamide	2',3'- (-OH) ₂	strong acid		(100, 101)
107	sec-butylidene-			2',3'- (-OH) ₂	methyl-ethylketone, (di)ethylphosphorothioate, 2,2-dimethoxypropane, dimethylformamide	2',3'- (-OH) ₂	strong acid		(100, 101)
108	1,3-dimethyln-butylidene-			2',3'- (-OH) ₂	methylisobutylketone, (di)ethylphosphorothioate, 2,2-dimethoxypropane, dimethylformamide	2',3'- (-OH) ₂	strong acid		(100, 101)

Table 1.1 (continued)

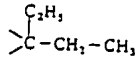
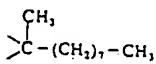


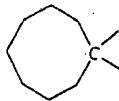
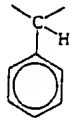
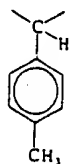
No. Blocking group	Abbrev- iation	Structural formula	Blocked moiety	Conditions for blocking	Deblocked moiety	Deblocking conditions	Special applications	References
109 1-ethyl- n-propylidene			2',3'- (-OH) ₂	diethyl- ketone, (di)ethyl- phosphoro- thioate, 2,2-di- methoxy- propane, dimethyl- formamide	2',3'- (-OH) ₂	strong acid		(100, 101)
110 1-methyl- n-nonylidene-			2',3'- (-OH) ₂	methyl- n-octyl- ketone, (di)ethyl- phosphoro- thioate, 2,2-di- methoxy- propane, dimethyl- formamide	2',3'- (-OH) ₂	strong acid		(100, 101)
111 Cyclopentyl- idene-			2',3'- (-OH) ₂	cyclopenta- none, HCl	2',3'- (-OH) ₂	strong acid		(100, 101, 197)
112 cyclo- heptylidene-			2',3'- (-OH) ₂	cyclohepta- none, HCl	2',3'- (-OH) ₂	strong acid		(197)
113 cyclo- octylidene			2',3'- (-OH) ₂	cyclo- octanone, HCl	2',3'- (-OH) ₂	strong acid		(197)
114 benzylidene			2',3'- (-OH) ₂	benzal- dehyde, p-toluene- sulfo- acid	2',3'- (-OH) ₂	acid		(99, 197)
115 p-methyl- benzylidene			2',3'- (-OH) ₂	p-methyl- benzal- dehyde, (di)ethyl- phosphoro- thioate, 2,2-di- methoxy- propane, dimethyl- formamide	2',3'- (-OH) ₂	acid		(100, 101)

Table 1.1 (continued)

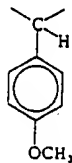
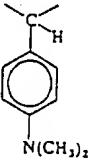
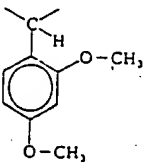
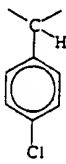

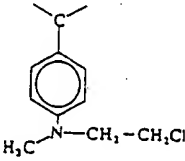
No. Blocking group	Abbrev- iation	Structural formula	Blocked moiety	Conditions for blocking	Deblocked moiety	Deblocking conditions	Special applications	References
116 4-methoxybenzylidene			2',3'-(OH) ₂	4-methoxybenzaldehyde, acid	2',3'-(OH) ₂	acid		(197)
117 4-dimethylaminobenzylidene			2',3'-(OH) ₂	4-dimethylaminobenzaldehyde, trifluoroacetic acid	2',3'-(OH) ₂	mild acid		(197)
118 2,4-dimethoxybenzylidene			2',3'-(OH) ₂	2,4-dimethoxybenzaldehyde, trifluoroacetic acid	2',3'-(OH) ₂	mild acid		(197)
119 4-chlorobenzylidene			2',3'-(OH) ₂	4-chlorobenzaldehyde, acid	2',3'-(OH) ₂	acid		(197)
120 p-nitrobenzylidene			2',3'-(OH) ₂	p-nitrobenzaldehyde, trifluoroacetic acid	2',3'-(OH) ₂	acid		(484)
121 p-(N-methyl-N-β-chloroethyl)-amino benzylidene			2',3'-(OH) ₂	p-(N-methyl-N-(β-chloroethyl)-amino)benzaldehyde, p-toluene-sulfonic acid	2',3'-(OH) ₂	mild acid		(118)

Table 1.1 (continued)

No.	Blocking group	Abbreviation	Structural formula	Blocked moiety	Conditions blocking	Deblocked moiety	Deblocking conditions	Special applications	References
<i>orthoesters and deriv.</i>									
122	methoxy-methylidene			2',3'- (-OH) ₂	trimethyl-ortho-formate, p-toluene-sulfonic acid	1. 2',3'- (-OH) ₂ 2. 2'(3')- -OH	1. mild acid→ formate 2. alkali		(117, 352)
123	ethoxy-methylidene concurrent with p-nitro-benzylidene			2',3'- (-OH) ₂ + -NH ₂	p-nitro-benzaldehyde + ethylortho-formate, trifluoro-acetic acid	1. 2',3'- (-OH) ₂ 2. 2'(3')- -OH -NH ₂	1. mild acid→ formate 2. alkali, acid		(479, 484)
124	dimethoxy-methylidene			2',3'- (-OH) ₂	tetramethyl-ortho-carbonate, p-toluene-sulfonic acid	1. 2',3'- (-OH) ₂ 2. 2'(3')- -OH	1. mild acid→ carbonate 2. alkali		(197)
125	methoxy-ethylidene			2',3'- (-OH) ₂	dimethyl-ortho-acetate, p-toluene-sulfonic acid	1. 2',3'- (-OH) ₂ 2. 2'(3')- -OH	1. mild acid→ acetate 2. alkali		(99, 352)
126	methoxy-benzylidene			2',3'- (-OH) ₂	trimethyl-ortho-benzoate, p-toluene-sulfonic acid	1. 2',3'- (-OH) ₂ 2. 2'(3')- -OH	1. mild acid→ benzoate 2. alkali		(99)
127	phenyl-boronate			2',3'- (-OH) ₂	phenyl-boronic acid	2',3'- (-OH) ₂	propane-diol-1,3 in DMF/water		(84, 202, 203, 474, 475)

1.3.1. Protecting Groups for the Phosphate Moiety

Phosphate protecting groups can be introduced in three ways:

1. Reaction of an appropriate alcohol or amine with a nucleotide, using a condensing agent.

2. Reaction of a nucleoside with an activated phosphoric acid ester or amidate of the blocking agent, e.g. the respective phosphorodichloridate.
3. Addition of nucleotides to blocking reagents, which are unsaturated systems, e.g. diphenyldiazomethane.

Depending on the deblocking conditions phosphate protecting groups as well as alcohol and amino protecting groups can be classified into alkali-labile groups, acid-labile groups and others which are removable at near neutral pH by specific reagents. The mechanism of deblocking of phosphate residues can follow two pathways which are outlined in Figs. 1.3 and 1.4. In pathway 1 the blocking group is removed by

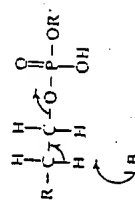


Fig. 1.3
R = blocking substituent
R' = nucleoside or oligonucleotide

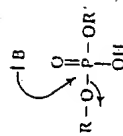


Fig. 1.4
R = blocking substituent
R' = nucleoside or oligonucleotide

rupture of the O-C_α-bond with liberation of a phosphate anion. Pathway 2 involves the attack of a nucleophile, in most cases water, on the phosphorus atom with rupture of the P-O-bond. This transfer of a phosphoryl moiety to another nucleophile is, in fact, the same as is used in the phosphorylation of alcohols (see Section 2.1). Alcoholysis generally necessitates a higher degree of activation, i.e. electron withdrawal from the phosphoryl moiety, but a sharp distinction between groups used for protection resp. activation is not possible (compare for example phosphoramidates and phosphoromorpholides). Thus,

References, pp. 483-508

in a few cases protecting groups have, indeed, been transformed into activating groups (see Section 1.4).

The most numerous and widely applied class of phosphate protecting groups is that of β-substituted ethyl esters (Table 1.1, no. 1-13). β-Substituents are introduced, which are electron withdrawing and allow alkaline cleavage with C-O-bond rupture according to Fig. 1.3 (for example R' = -CN for the β-cyanoethyl group). In the special case of groups no. 10 and 12 (phenylmercaptoethyl resp. uridylyl) the β-substituent has to be rendered electron-withdrawing by oxidation before the group becomes alkali labile (178, 290a). The trichloroethyl group (no. 13) is deblocked by reduction with a zinc/copper couple in a neutral medium, the zinc atom acting as electron donor instead of alkali (Fig. 1.5) (76, 78).

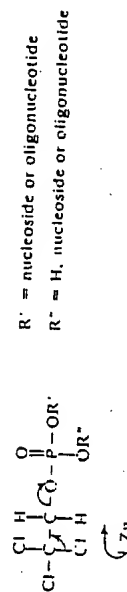
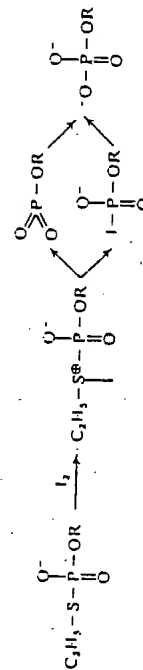


Fig. 1.5

Groups no. 14-25 are also ester groups, but their cleavage can involve liberation of either phosphate or phosphoryl groups. Of these the phenyl groups (no. 14-17) have become interesting for protection of the internucleotidic bond (see "triesther method", Section 4) (352). Three other groups of this series (no. 19, 20 and 23) offer a possibility of cleavage in neutral medium. Especially useful in polynucleotide synthesis is the ethylthio group (no. 23) which has been investigated by A. L. NUSSBAUM and his colleagues (55). Scission of the P-S-bond is effected by iodine oxidation with possible formation of a phosphoriodinate intermediate, which allows not only hydrolysis, but also alcoholysis (Scheme 1.6).



Scheme 1.6. Removal of the ethylthio group
R = 3'-O-acetylthymidine-5'

A route to a third class of phosphate protecting groups, namely the phosphoramidate type (no. 26—30) was opened up by the finding of E. OHTSUKA and coworkers, that acidic hydrolysis of phosphoranilates, normally accompanied by glycoside cleavage, can be carried out under very mild conditions, if isoamylnitrite is added to the medium (310). Similar to the ethylthiophosphate case, electrophilic attack on the nitrogen atom with intermediate nitrosation is believed to be responsible for the easy removal of this group. All phosphoranilate groups thus belong to the type of residue cleaved according to Fig. 1.4. By appropriate substitution of the anilinate residue several groups with special properties have been developed, useful, for example, for activation (no. 27) (316) solvent extraction (no. 29) (3) or affinity separation (no. 30) (136) of nucleotides or oligonucleotides.

1.3.2. Protecting Groups for the Hydroxyl and Amino Functions of the Sugar and Base Moieties

The introduction of blocking groups into the hydroxyl and amino functions of sugar and bases proceeds by reactions similar to the ones discussed for phosphate protection, namely

1. Reaction of activated derivatives of carboxylic acids, carbonic or carbamic acid and of highly electrophilic alkyl halides with sugar and/or bases,
2. Reaction of "activated alcohol derivatives" of nucleosides with protecting agents,
3. Acid-catalyzed acetalization, ketalization or transacetalization of nucleosides,
4. Addition of nucleosides and nucleotides to compounds with polarized double bonds.

By far the greatest number of protecting groups is attached by reaction type 1. Esterification of nucleotide hydroxyl and amino groups with acid chlorides or preferably anhydrides generally proceeds with excellent, often quantitative yields. The alternative route, reaction with a carboxylic acid and a condensing agent, is less attractive because yields are lower and is used only when activated acid derivatives are not available (e.g. benzoylpropionic acid, no. 44). Trityl and benzyl groups are similarly introduced by the action of trityl and benzyl halides.

Reaction 2 is still an exceptional case for alcohol protection. The activation of hydroxyl groups of the sugar moiety by triphenylphosphine and azodicarboxylate has been described by O. MITSUNOBU and coworkers (264). Activated alcohol groups can be reacted with carboxylic acids as well as with phosphoric acid (see Section 2.2); for steric reasons the 5'-OH group is specifically substituted. The blocking

References, pp. 483—508

of OH-groups by reaction of nucleoside chloroformates with alcohols and amines may, to a certain extent, also be counted among reactions of this type. On the whole, activation of the alcohol groups of nucleosides is an interesting addition to the possibilities of protection, which merits further investigation.

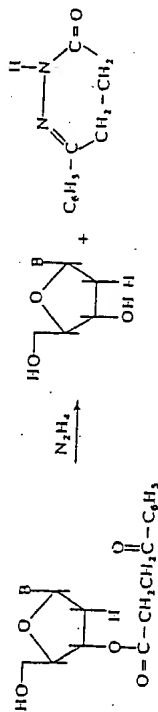
Examples of reactions with aldehydes, acetals and orthoesters are equally scarce in the protection of "isolated" OH- and NH₂-groups (for the blocking of vicinal diol groups see Section 1.3.3). The only case of importance is the formation of Schiff bases specifically with exocyclic amino groups of the bases by treatment with dimethylformamide acetals.

Reaction with enol ethers (type 4) leads to introduction of acetal and ketal groups, which are of great importance for the protection of ribo nucleosides and nucleotides. As acid catalysis is necessary for this reaction, their use is restricted to the ribo series.

A more detailed discussion of ester type protecting groups has to start with the acetyl and benzoyl groups (no. 33 and 52), both of which are standard protecting substituents in sugar chemistry. Acetylation is normally carried out with acetic anhydride, whereas the more reactive acyl halides are preferred for benzoylation. Treatment with these reagents normally leads to substitution of all OH- and NH₂-functions; partial deblocking of the hydroxyl groups can subsequently be effected by strong alkali under controlled conditions due to the relative stability of carboxamide relative to ester moieties at high pH (197). The conditions of these acylation reactions are subject to a great deal of variation, and some of these variations are noteworthy for reasons of selectivity. Thus, the amino group of cytosine nucleoside and nucleotide could be selectively acetylated by acetic anhydride in dimethylformamide (329, 330) and benzoylated by benzoic acid -N-hydroxysuccinimide ester (303). The 5'-hydroxyl-selective acylation by, for example, benzoic acid, triphenylphosphine and azodicarboxylate (264) was discussed above. Selective acylation of either 2'- or 3'-OH in ribosides or ribotides can be achieved by acid treatment of ortho-ester derivatives (no. 125 and 126) (99). Suitable substitution of the acetyl or benzoyl substituent gives blocking groups which are more readily (e.g. no. 34, 35, 36, 37 and 54) or less readily (e.g. no. 46—49, 53) cleaved by alkali than the parent groups. The first alternative is preferred in oligoribonucleotide synthesis; the second is sought for in the design of amino protecting groups for deoxyoligonucleotides. The methoxyacetyl, isobutyl and anisoyl groups are among the most widely used in oligonucleotide synthesis.

Ester groups, which offer a possibility of cleavage in neutral medium, are the chloroacetyl (57) resp. benzoylpropionyl and benzoylformyl

groups (230, 234) (no. 32, 38, 44), all displaced by a cyclisation mechanism with thiourea or 2-mercaptoethylamine in the case of no. 38 and hydrazine resp. *o*-phenylenediamine in the two latter cases (Scheme 1.7). Hydrazine treatment in a pyridinium acetate buffer



Scheme 1.7. Removal of the benzoylpropionyl group

will also selectively remove benzoyl or anisoyl groups from the nucleobase (233). The 2,4-dinitrobenzenesulfonyl group is readily cleaved in a neutral medium by thiophenol (197), however, its use is restricted by alkali-sensitivity. Similarly, the formyl group, although very useful in polypeptide synthesis, has not been widely used in the polynucleotide field due to its extreme lability in weakly alkaline media. Recent investigations, however, show, that 3'-O-formyl esters of deoxynucleotides and -nucleoside polyphosphates can be readily synthesized in quantitative yield by action of formic acid or formic acetic anhydride on base-protected nucleotides and directly used for oligonucleotide synthesis without purification. The formyl group is stable in pyridine solution and is lost during aqueous workup of the condensation mixture (332, 398).

A completely different approach to selective cleavage in neutral medium, namely the enzymatic hydrolysis by esterases, has been investigated by A. TAUNTON-RIGBY and N. A. STARKOVSKY for the case of the dihydrocinnamoyl substituent (no. 43) (363).

Blocking groups of the carbonate or carbamate type are among the most widely used in the peptide field, and a number of such groups has also been of use in oligonucleotide synthesis (no. 61—69). Of these the isobutyloxycarbonyl group (no. 64) can be selectively attached to the 5'-hydroxyl group due to steric hindrance (236, 304); the trichloro- or tribromophenylloxycarbonyl group can be cleaved in near neutral medium by Zn/Cu in methanol/acetic acid by a mechanism similar to the one described in Fig. 1.5, section 1.3.1 (54, 183, 466). It appears that the full scope of possibilities for selective introduction and cleavage is not yet fully exploited for this class of protecting groups in the nucleotide field. The possibility of introducing a variety of alcohol or amine substituents by addition to nucleoside chloroformates

References, pp. 483—508

(391) may simplify the search for new solutions. An interesting new development in this area is the naphthylcarbamoyl group (no. 69), described by K. L. AGARWAL *et al.* (4) for the blocking of unreacted 3'-OH ends by a group, which permits separation of truncated sequences by affinity chromatography (see Section 1.4).

Orthoester groups, widely used for the blocking of the vicinal diol function in ribonucleosides (see 1.3.3) are occasionally also employed for the protection of other hydroxyl and amino functions. Of the examples given in Table 1.1 (no. 71—73) the dimethylaminomethylidene group (no. 72) is of special interest, because it is the only group which can be selectively introduced into the base (197). Its application in oligonucleotide synthesis is, however, limited by the fact that it is labile to mild acid as well as alkaline conditions. Dimethylformamide dimethyl- and di-n-pentyl acetal are generally used to introduce this group. The second reagent is preferred in many cases, since the dimethyl acetal can also act as an alkylating (481) or dephosphorylating agent (480).

A second major category of hydroxyl and amino blocking groups (no. 74—84) includes those which have ether, acetal and ketal substituents. Benzyl ethers have been used for hydroxyl protection since the beginnings of oligonucleotide chemistry; however, their use is restricted by the fact that deprotection through catalytic hydrogenation may affect the pyrimidine bases (258). Apart from this group all other ether substituents are of the trityl type. As cleavage of unsubstituted trityl ethers requires relatively strong acidic conditions, e.g. 80% acetic acid for several hours at reflux, *p*-methoxy substituents have been introduced to facilitate their removal. Each *p*-methoxy substitution produces a ten-fold acceleration of the rate of acidic hydrolysis, as was found by chromatographic (6) and NMR (354) investigations. Mono- and di-*p*-methoxytrityl groups are at optimum as to stability and deblocking conditions and are widely used in polynucleotide synthesis, especially as, under appropriate conditions, they are selectively introduced into the 5'-hydroxyl group of N-protected nucleosides and nucleotides (184, 197).

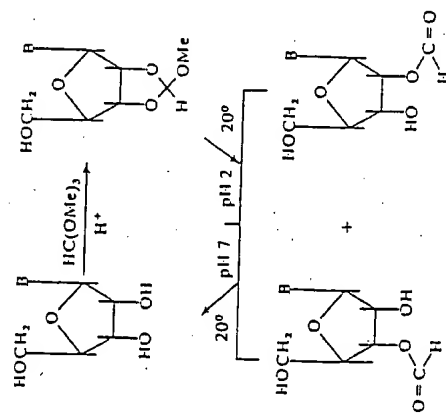
Acetal and ketal groups (no. 85—99) need acid catalysis for introduction and removal and, therefore, are more suitable for work in the ribo series. Several acyclic and cyclic alkyl vinyl ethers have been employed as blocking agents. The ethoxyethyl- (197), tetrahydropyranyl- (197) and methoxytetrahydropyranyl groups (352) (no. 86, 96 and 97) have been successfully employed in oligoribonucleotide synthesis for several years. Recently the methoxytetrahydrothiopyranyl group (no. 98) has been described; its acid stability can be regulated by oxidation of the sulfide moiety (32). Methoxyethyl groups (no. 85) have been introduced

for the purpose of enzymatic monoaddition (252) (see Section 4). Since the cytosine base is protonated under the conditions of reaction with dihydropyran, this could be used to selectively introduce the tetrahydropyranyl group into the 3'-position of deoxycytidine monophosphate as an intermediate step in the preparation of N-benzoyl-deoxycytidylic acid (197). This is one of the few examples, in which such blocking groups have been applied in the deoxy series.

1.3.3. Protecting Groups for the Vicinal Diol Group of Ribonucleic Acid Constituents

Acetal, ketal and orthoester substituents (no. 100—127) are used for protection of the 2,3'-diol function in ribotides. They are generally introduced by acid-catalyzed reaction of the diol group with appropriate aldehydes, ketones or orthoesters. Isopropylidene groups (no. 100), standard protecting agents in sugar chemistry, are often used (197). However, they are very stable to acidic hydrolysis, and in order to avoid an isomerization of the internucleotidic bond more acid-labile groups, such as 2,4-dimethoxybenzylidene and 4-dimethylaminobenzylidene (no. 117 and 118) (197) are preferred for stepwise oligoribonucleotide synthesis.

The orthoester substituents (no. 122—126) possess the additional feature of undergoing isomerization to 2'- or 3'-acylates on mild



Scheme 1.3. Removal of orthoester substituents

References, pp. 483—503

treatment with aqueous acid (117, 352). This offers a possibility for differentiation between the two hydroxyl groups which are of equal reactivity in internucleotide bond formation (for further discussion see Section 1.6). Final removal of these protecting groups then necessitates mild alkali or ammonia treatment (Scheme 1.8).

One example of a diol protecting group which can be removed in a neutral medium, has been reported: the phenylboronate substituent (no. 127), described by A. M. Yurkevitch and coworkers (475). It is easily introduced by treatment with phenylboronic acid. Cleavage is effected by propanediol-1, in dimethylformamide. Although this group is stable during the formation of internucleotidic bonds, its lability in aqueous acidic to neutral media is disadvantageous, since the diol protection must be stable throughout all steps of a ribooligonucleotide synthesis (see Section 1.6).

In concluding this section it should, again, be emphasized that alternatively, the diol moiety can be blocked by two substituents of the type discussed in Section 1.3.2, e.g. by two acyl groups. Several investigators (see Section 1.6) have employed this as the method of choice for oligoribonucleotide synthesis.

1.4. Protecting Groups with Special Applications

In several more recent publications blocking groups have been described which perform an additional job, such as opening up new ways of separation or activating the nucleotides or oligonucleotides to which they are attached. Some of the most significant developments in this area will be highlighted in this section.

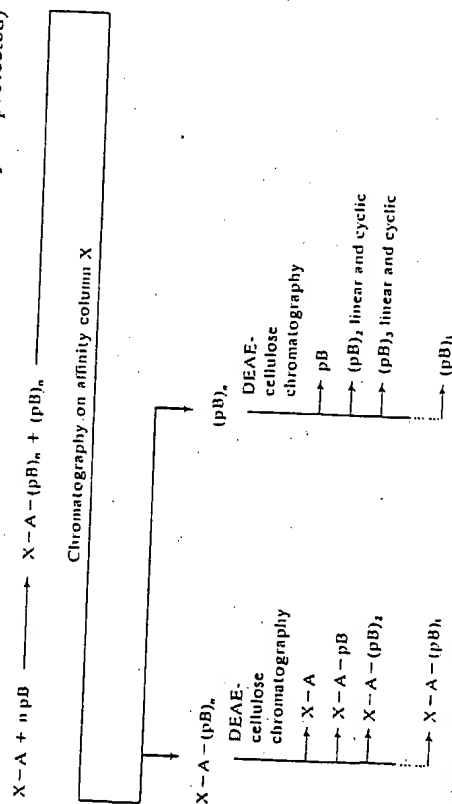
1.4.1. Protecting Groups for Solvent Extraction

The introduction of a blocking group will in most cases change the solubility properties of polynucleotides and their constituents, a change which most often goes in the direction of enhanced hydrophobicity. In extreme cases nucleotides or oligonucleotides can become water-insoluble and extractable into water-immiscible organic media. Examples have been described of oligonucleotide synthesis using *p*-aminophenyltriphenylmethane (3) or 9-fluorenylmethanol (176) for protection of the phosphate component (groups no. 11 and 29). The use of these groups in the preparation of short-chain oligonucleotides will be discussed in more detail in Sections 3.2 and 4.1.1.

1.4.2. Protecting Groups for Separations by Affinity Chromatography

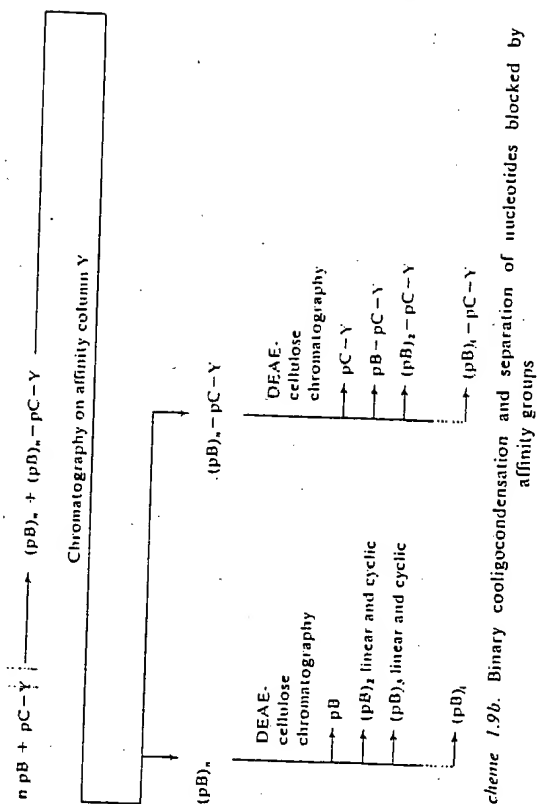
Mono-, oligo- and polynucleotides with hydrophobic substituents were shown to be retained on appropriate affinity columns. Chromatographic materials used for this purpose are, for example, tritylated or naphthoylated cellulose (41) or benzoylated DEAE cellulose (259, 289). The demands placed on the hydrophobicity of groups for this technique are not as restricting as for the solvent extraction procedures; this permits, in principle, the use of a wide variety of substituents for such separations. H. G. KHORANA and coworkers, for instance, have separated tritylated oligonucleotides from untritylated oligomers and monomers on trityl cellulose. Similarly, homologs of shorter chain length are removed during stepwise oligonucleotide synthesis after protection of their free 3'-OH ends by naphthylisocyanate (no. 69) (4, 6). Hydrophobic protecting groups, mostly of the β -substituted ethyl phosphate type (no. 7-10), have been studied by S. A. NARANG and coworkers for stepwise oligonucleotide syntheses with purification on benzoylated DEAE (289, 290, 290a, 465) or benzoylated DEAE-Sephadex (259). Affinity separations, based on the interaction of oligonucleotides blocked with the *p*-dimethylammoniummanilide residue (no. 30) with cation exchangers, have been employed for stepwise oligonucleotide synthesis by T. HATA *et al.* (136, 138, 435).

A different approach has been studied by H. SELIGER and coworkers (396, 398a, 398b). When nucleotides or nucleosides, blocked by affinity groups, are copolymerized with unprotected (resp. only N-protected)



Scheme 1.9a. Binary colligococondensation and separation of nucleotides blocked by affinity groups

References, pp. 483-508



Scheme 1.9b. Binary colligococondensation and separation of nucleotides blocked by affinity groups

nucleotides, two types of homologous sequences are produced, namely those that bear affinity end groups and others that do not. Two types of affinity groups have been used, namely hydrophobic groups, such as methoxy trityl or phenylazophenylcarbonyl (no. 67 and 76) for the 5'-terminus and the uridyl group (no. 12) for the 3'-terminus. Separations are carried out on trityl cellulose resp. boronate celluloses. A general reaction and separation scheme for binary copolymerizations of the components X-A and pB resp. pB and pC-Y (where X and Y are affinity groups and A, B and C are nucleosides) is given in Scheme 1.9. The two types of homologs, X-A-B_n and B_n resp. B_n and B_n-C-Y are subsequently separated into individual sequences according to charge, thus yielding a variety of building fragments for polynucleotide synthesis through one reaction.

1.4.3. Activable Protecting Groups

In the discussion of phosphate protecting groups (Section 1.3.1) we have pointed out that there may be no fundamental difference between protecting and activating residues. Hence, several phosphate protecting groups can be modified to become strongly activating groups, thus allowing alcoholic and phosphorolytic attack on the phosphorus atom. Early work by F. CRAMER and H. SELIGER (399) showed that functions, such as enol esters on phosphoric acids become strongly activating on

bromination. Similar results with nucleotides have been obtained only recently. Thus, the ethylphosphorothioate group (no. 23) on oxidation with iodine yields an activated intermediate, which can undergo alcoholysis (55) (compare Scheme 1.6). Oxidative activation was also demonstrated for the *p*-hydroxyanilinate residue (no. 27) by E. OHTSUKA *et al.* (316) (see Scheme 4.21). In this case internucleotide linkages could be formed on activation in presence of a nucleoside, although in moderate yield. The 4-nitro-2-chloromethylphenyl residue (no. 19) is activated in the presence of tertiary bases (e.g. pyridine) by quaternization (281).

A fourth area of special application of blocking groups, namely in the formation of enzymatic monoaddition substrates, is reviewed in more detail in Section 5.1.

1.5. Strategy of Consecutive Blocking or Deblocking of Several Functions

The last two sections of this chapter deal with different approaches to the preparation of intermediates for nucleotide polycondensation and stepwise oligonucleotide synthesis. Initially some general considerations governing the choice of blocking groups for certain functions will be discussed.

We have seen in earlier sections, that differences in nucleophilicity between the hydroxyl groups of the sugar and the exocyclic amino groups of the bases are relatively small. This means that, except for the reaction of nucleotide phosphate groups with alcohols and amines as blocking reagents, all other reactions used for the introduction of blocking groups tend to be unspecific. Nevertheless, it is possible to block selectively one of several alcohol or amino functions by almost any group by adopting one of the following routes:

1. If a selective reagent is available, this can be directly used to block the desired function.
2. If a selective reagent is available for the other functions, these can be blocked first. The function in question is left free to react with an unspecific reagent. Afterwards the other blocking groups are removed, if necessary.
3. An unspecific reagent is used to introduce a blocking group into all functions. All but the desired function are then selectively deblocked.
4. If this is not possible, total blocking can be followed by selective deblocking of the desired function, which is then reblocked with the reagent of choice.

References, pp. 483—508

These four routes can be illustrated for the example of the acetyl protecting group:

Route 1: Selective acetylation of the 5'-OH group is affected by acetic acid / azodicarboxylate / triphenylphosphine (264). For the exocyclic amino group of the cytosine nucleotides acetic anhydride in dimethylformamide / tri-*n*-butylamine has been described as selective reagent (329, 330). Finally, 2' or 3'-OH groups of ribonucleosides can be acetylated with trimethylorthoacetate *via* a methoxyethylidene intermediate (99).

Route 2: The selective 3'-O-acetylation of nucleosides has been described by introducing first a dimethylaminomethylene group into the base, then a methoxytrityl group into the 5'-hydroxyl. Acetylation followed by acid treatment leaves acetyl as only protecting group in 3'-position (197).

Route 3: The most widely used approach to N-acetylation of nucleosides and nucleotides involves unspecific reaction with acetic anhydride in pyridine, followed by selective removal of O-acetyl groups on short treatment with strongly alkaline media (197).

Route 4: N-benzoyl-3'-O-acetyl nucleotides are usually synthesized by perbenzoylation of nucleotides, followed by selective O-debenzoylation in strong alkali and treatment with acetic anhydride (184, 197).

A detailed description of all possibilities for selective introduction or removal of the different blocking groups listed in Table 1.1 would by far exceed the scope of this review. We must limit ourselves to some general observations. Thus, for example, sterically hindered reagents react more readily with amino and 5'-hydroxyl groups than with 2'- and 3'-hydroxyl. The rate of hydrolytic cleavage can also be higher for 5'- than 3'-substituents as was demonstrated for nucleosides containing several trityl groups. In the case of acylations the reaction conditions may be chosen so as to give specific substitution of the nucleobase in cytidylic acid. The 3'- resp. 2'-OH groups as sterically most hindered functions can generally not be blocked in neutral or weakly basic media without prior protection of the amino and (if unphosphorylated) 5'-hydroxyl groups. More possibilities for selective reactions are contained in Table 1.1, and the information given there may be of help in designing new approaches.

1.6. General Blocking Schemes for Intermediates of Polynucleotide Synthesis

The basis for chemical synthesis of oligo- and polynucleotides, as of other complex organic molecules, is the adoption of a certain strategy for protecting the intermediates. Since the preparation of sufficient

quantities of blocked intermediates constitutes a major part of the work involved in the synthesis of longer oligo- and polynucleotides, the groups working in this field tend to pursue one strategy, once they have developed it, for the duration of one or several synthetic projects, like for example, the synthesis of a biologically important polynucleotide. Evidently most groups which tackle the preparation of a polynucleotide have started out by developing a new technique, often a new protecting group or a system of protecting groups. Although this means, that most groups have a different approach and that the authorship of a sequence can often be predicted from the type of intermediates used in the synthesis, some generalizations can be made and will be discussed in the following.

In planning the synthesis of a deoxyoligonucleotide we first have to decide whether the diester or triester method shall be used (see Section 4.1). In the diester method 5'-nucleotides are mostly taken for chain extension, nucleosides only as terminal units. The triester method allows chain elongation reaction with a blocked phosphomonoester, followed by condensation with a nucleoside. The advantage of the latter approach in the sector of blocking groups is that only one type of intermediate is needed for terminal and intrachain units and nucleosides are generally cheaper starting compounds. However, the large-scale preparation of N,O^3' -protected nucleosides is more time-consuming than the synthesis of analogous nucleotide derivatives, as is clear from the preceding section.

Next the blocking groups for nucleotides and nucleosides have to be selected. Both kinds of monomer units contain three functions, two of which have to be blocked for stepwise oligonucleotide synthesis, namely either

5'-OH resp. -phosphate and bases

3'-OH resp. -phosphate and bases.

In an ideal situation these three functions would have to be blocked by three independently-removable blocking groups. This has not yet been realized. Although there are, in principle, three types of de-blocking conditions, namely alkaline, acid and neutral with selective reagents, the choice is limited by the circumstance that acid labile groups are preferred as end groups only (deglycosidation hazard, see Section 1.2) and selectively cleavable groups are mostly also acid or alkali-labile. One feature is common to all approaches: The amino groups of the nucleobases are protected most strongly, usually by a group which is removed only by prolonged alkaline treatment. Benzoyl and anisoyl have been widely used for deoxyadenosine resp. deoxycytidine and their

References, pp. 483-503

nucleotides, acetyl and more recently isobutyryl, isobutyloxycarbonyl, methylbutyryl and also benzoyl have been advocated for deoxyguanosine and dGMP (6, 184, 197, 205, 315, 457).

For the protection of phosphate and hydroxyl functions three different strategies have been generally applied.

1. The strategy developed mainly by H. G. KHORANA and coworkers (6, 184, 197, 319, 457) uses preferably compounds A of Table 1.2 as intermediates. The methoxy- or dimethoxytrityl group serves as acid-labile 5'-end group. The 5'-phosphate group of nucleotides is protected by the β -cyanoethyl moiety, labile to brief treatment in alkali. The acetyl group, removable by alkali or ammoniacal treatment, is taken for 3'-end protection. Thus, 5'-terminal units or building fragments for polynucleotide synthesis contain an acid-labile and an alkali-labile end.

Chain extension is effected by monomers of building blocks containing a 5'-phosphate and a 3'-hydroxyl end. All these building fragments such as a dinucleotide (Fig. 1.12), on construction from units

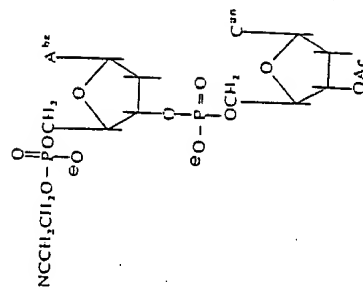


Fig. 1.12

A 1+2 of Table 1.2, will have two alkali-labile blocking groups at the ends. Their removal leaves both ends free. Reblocking by acetylation gives a new building block for lengthening towards the 3'-end, β -cyanoethylation affords a fragment for lengthening towards the 5'-end. This strategy has been used successfully throughout the work on the synthesis of two genes and is described explicitly by H. BÜCHER, H. WEBER and H. G. KHORANA (37, 457).

Table 1.2. Some Examples of Blocked

Method	5'-terminal units	I'
A	Fig. 1.10 R_1 = methoxy-trityl- = dimethoxy-trityl- R_2 = acyl- R_3 = H-	
B	Fig. 1.10 R_1 = methoxy-trityl- = dimethoxy-trityl- R_2 = acyl- R_3 = H-	
C	Fig. 1.10 R_1 = methoxytrityl- R_2 = acyl- R_3 = H- \longrightarrow R_3 = OH - P=O O-CH ₂ -CH ₂ -CN	Fig. 1.10 R_1 = methoxytrityl- R_2 = acyl- R_3 = OH - P=O O-CH ₂ -CH ₂ -CN
D*	Fig. 1.10 R_1 = trityl- R_2 = H- \longrightarrow R_3 = O-H - P=O O-CH ₂ -CCl ₃	Fig. 1.10 R_1 = trityl- R_2 = O-H - P=O O-CH ₂ -CCl ₃
E*	Fig. 1.10 R_1 = 2,4-(bis-2-methyl- butyl-2-)phenyl- oxyacetyl- R_3 = H- \longrightarrow R_3 = OH - P=O O-C ₆ H ₅	Fig. 1.10 R_1 = 2,4-(bis-2-methyl- butyl-2-)phenyl- oxyacetyl- R_3 = OH - P=O O-C ₆ H ₅

* R_2 is not given, where thymidine derivatives were the only ones used.

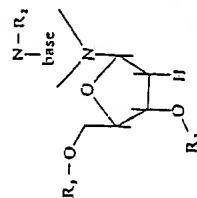


Fig. 1.10

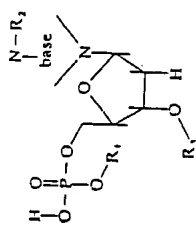


Fig. 1.11

Intrachain units:	2	3'-terminal units:
Fig. 1.11 $R_1 = \beta$ -cyanoethyl- $R_2 = \text{acyl-}$ $R_3 = \text{H-}$		Fig. 1.11 $R_1 = \text{H-}$ $R_2 = \text{acyl-}$ $R_3 = \text{acetyl-}$
Fig. 1.11 $R_1 = \text{trichloroethyl-}$ = amidate, = ethylthio-, = phenylmercapto-ethyl- $R_2 = \text{acyl}$ $R_3 = \text{H-}$		Fig. 1.11 $R_1 = \text{H-}$ $R_2 = \text{acyl-}$ $R_3 = \text{acetyl-}$
Fig. 1.10 $R_1 = \text{H-}$ $R_2 = \text{acyl-}$ $R_3 = \beta\text{-benzoyl-}$ propionyl-	Fig. 1.10 $R_1 = \text{H-}$ $R_2 = \text{acyl-}$ $R_3 = \beta\text{-benzoyl-}$ propionyl- -P=O O-CH ₂ -CH ₂ -CN	Fig. 1.10 $R_1 = \text{H-}$ $R_2 = \text{acyl-}$ $R_3 = \text{methoxytrityl-}$ = β -benzoyl-propionyl-
Fig. 1.10 $R_1 = \text{H-}$ $R_2 = \text{acyl-}$ $R_3 = \text{acetyl-}$	Fig. 1.10 $R_1 = \text{H-}$ $R_2 = \text{acyl-}$ $R_3 = \text{OH}$ -P=O O-CH ₂ -CCl ₃	same as intrachain unit D ₂
Fig. 1.10 $R_1 = \text{H-}$ $R_3 = \text{methoxy-}$ tetrahydro- pyranyl-	Fig. 1.10 $R_1 = \text{H-}$ $R_3 = \text{OH}$ -P=O O-C ₆ H ₅	same as interchain unit E ₂

Recent Advances in Polynucleotide Synthesis

with selectively unblocked 3'-OH. Several routes to the latter compounds have been described.

In a different method elaborated by the group at Prague (152) the permanently blocked positions (2'-OH and terminal diol) contain acid-labile groups. Since acid-labile protection of the bases is unusual, the amino groups and the 5'-hydroxyl function in intermediate H 1 (Table 1.3) are protected in an alkali-labile fashion. Chain lengthening is again done from the 3' to the 5'-terminus. On deblocking of the 5'-end both alkali-labile groups are lost and the NH₂-functions can be selectively reprotected by treatment with dimethylformamide acetals. The intermediates H 2 and H 3 are also N-dimethylaminomethylene

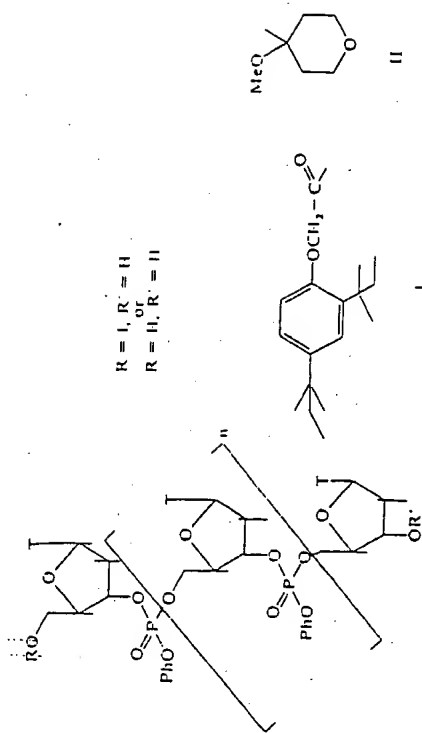
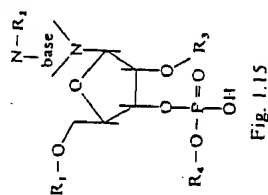


Fig. 1.13

In the synthesis of oligoribonucleotides an additional complication is introduced by the presence of the 2'-OH group. Since the hydroxyl groups at the 2'- and 3'-position are about equally reactive towards electrophilic attack some kind of differentiation is necessary to form intermediates which allow an internucleotidic bond to be formed specifically at O-C3'. This is most simply achieved by using suitably blocked nucleoside-3'-phosphates, which are available either by substitution of 3'-ribonucleotides or by phosphorylation of ribonucleosides (73).

Intermediates for Oligoribonucleotide Synthesis

Intrachain units:	2'	3'-terminal units:
<p>Fig. 1.15 $R_1 = \text{trityl-}$ $R_2 = \text{acyl-}$ $R_3 = \text{acyl-}$ $R_4 = \text{H-}$</p>	<p>same as 3'-terminal unit H_1.</p>	<p>Fig. 1.14 $R_1 = \text{H-}$ $R_2 = \text{benzoyl-}$ $R_3 = \text{benzoyl-}$ $R_4 = \text{benzoyl-}$</p>
<p>Fig. 1.15 $R_1 = \text{H-}$ $R_2 = \text{acyl-}$ $R_3 = \text{acyl-}$ $R_4 = \text{anilide-}$</p>	<p>Fig. 1.14 $R_1 = \text{H-}$ $R_2 = \text{dimethylamino-methylene-}$ $R_3 = \text{ethoxyethyl-}$ $R_4 = \text{H-}$</p>	<p>Fig. 1.15 $R_1 = \text{H-}$ $R_2 = \text{acyl-}$ $R_3 = \text{acyl-}$ $R_4 = \text{anilide-}$</p>
<p>Fig. 1.14 $R_1 = \text{H-}$ $= -\text{PO}_3\text{H}_2$</p>	<p>Fig. 1.14 $R_1 = \text{H-}$ $R_2 = \text{acyl-}$ $R_3 = \text{methoxytetrahydropyranyl-}$ $R_4 = \text{H-}$</p>	<p>Fig. 1.14 $R_1 = \text{H-}$ $R_2 = \text{acyl-}$ $R_3 = \text{methoxymethylidene-}$ $R_4 = \text{methoxymethylidene-}$</p>
<p>Fig. 1.14 $R_1 = \text{H-}$ $R_2 = \text{acyl-}$ $R_3 = \text{tetrahydropyranyl-}$ $R_4 = \text{H-}$</p>	<p>Fig. 1.14 $R_1 = \text{H-}$ $R_2 = \text{acyl-}$ $R_3 = \text{tetrahydropyranyl-}$ $R_4 = \text{H-}$</p>	<p>same as intrachain unit K_2</p>



derivatives. The phosphate-blocked intermediate H 1' was recently used by J. SMIT for triester syntheses (418, 419). Condensation of H 1' with H 3 gave a fully protected dinucleoside phosphate, from which the 5'-blocking group was selectively cleaved prior to chain lengthening, which was done again with H 1'.

C. B. REESE and coworkers (352) have extensively studied the synthesis of intermediates for oligoribonucleotide synthesis in the diester and more recently also in the triester fashion. The units I of Table 1.3 are proposed as intermediates for stepwise oligoribonucleotide synthesis. They all contain acyl or methoxytetrahydropyranyl groups for the bases resp. 2'-OH. The methoxymethylidene group is generally employed to block the diol end. In the diester approach the chain is lengthened by adding units I 2 to the 3'-end of the 5'-terminal unit or fragment. For syntheses by the triester variation the 3'-end is first reacted with phenyl- or substituted phenyl phosphate, then the oligonucleotide is extended with intermediates I 2' or I 3.

Further improvements were introduced by T. NEILSON and coworkers (296, 298). They use exclusively the triester method, starting the synthesis from the 5'-end, which is blocked by the trityloxyacetyl moiety. The chain is lengthened by first adding trichloroethylphosphate, then the intermediates K 2 of Table 1.3. Most remarkably these intermediates are unblocked at the 3'-position, since it was found that the bulky nucleoside-3'-trichloroethyl phosphate would not react with the 3'-OH of another nucleoside, due to "shielding" by the neighbouring tetrahydropyranyl group. This triester approach has been the first one leading to the synthesis of a longer oligoribonucleotide chain (300), as will be discussed in Section 4.1.2.

In concluding this section it should be made clear that the intermediates discussed here are only examples of some more widely used blocking schemes. Nearly all of the blocking groups listed in Table 1.1 have been tested during the "evolution" of one or the other system of selective blocking, and it is impossible, in this review, to retrace all the different lines of development. In polynucleotide, as in polypeptide synthesis, the search for better and more straightforward solutions continues. New selectively labile protecting groups are still much in demand, and the possibility of enzymatic cleavage is a further valuable addition in this sector. New blocking groups for nucleobases stable to all conditions of polynucleotide synthesis and selectively removable in neutral medium would be helpful. New developments in the direction of "multipurpose" blocking groups are to be foreseen, and it can be hoped, that these developments will lead to simpler solutions not only for the field of protection, but for all questions involved in polynucleotide synthesis.

References, pp. 483—508

2. Phosphorylation Methods in the Synthesis of Mono- and Oligonucleotides

Polynucleotides are — from the standpoint of polymer chemistry — polyphosphodiester. All internucleotide linkages are thus phosphodiester groups, whereas the chain termini can be either nucleosides or phosphomonoesters. The formation of a phosphoric acid ester linkage, i.e. the phosphorylation, can be effected chemically

1. by transfer of a phosphoryl group onto an alcohol with formation of a P—O—bond,
2. by transfer of a phosphate group onto an alcohol with formation of a C—O—bond,
3. by oxidation of a phosphite.

Phosphorylation by phosphoryl transfer is still the most widely used phosphorylation method in the synthesis of nucleotides and oligonucleotides. Reagents and mechanisms for phosphoryl transfer have been very extensively studied in the early 1960s. Since the development of the common phosphorylation techniques by H. G. KHORANA, F. CRAMER and others nearly ten years ago, no major advance in this field has been reported, and as several excellent reviews of this earlier work have appeared (35, 61, 65, 70, 184, 253, 451) we can limit ourselves to giving a few guidelines on the selection of phosphorylating agents and a brief mechanistic discussion.

In recent years an increasing number of instances have been described, where not a phosphoryl, but a phosphate group is transferred, as, for example, during studies of prebiotic or "thermal" phosphorylations and of phosphate transfer to activated nucleoside hydroxyl groups. Although phosphoryl transfer is still the route generally employed in internucleotide bond formation, this mechanistically different approach certainly merits continued interest. The same can be said of phosphorylations involving the oxidation of a nucleoside phosphite. Although this is one of the oldest phosphorylation methods, it has not furnished a breakthrough for internucleotide bond formation. Nevertheless, this pathway is often reinvestigated and results in the development of new reagents and techniques.

An elegant route, if applicable, is enzymatic attachment of a phosphoryl moiety. Since enzymatic methods of internucleotide bond formation will be described in Section 5 the discussion in Section 2.4 can be limited to the description of several kinases.

2.1. Transfer of a Phosphoryl Group

The transfer of a phosphoryl group to water, alcohols or amines proceeds through nucleophilic attack of these compounds on the phosphorus atom of a phosphorylating agent. Before we discuss the question of what should be defined as a phosphorylating agent, we should first clarify some basic steric and electronic aspects of phosphate chemistry.

In orthophosphoric acid and its derivatives the phosphorus atom occupies the centre, the four ligands the corners of a tetrahedron (16f). Nucleophilic substitution reactions on phosphorus can be described in the same way as nucleophilic substitutions on a saturated carbon atom. Thus, there are two principal mechanistic pathways. The first, similar to the S_N2 reaction on carbon, involves direct attack by the nucleophile on the phosphorus atom with displacement of one of the four ligands. This mechanism must involve an inversion of the ligands with intermediacy of a pentacoordinate complex. Alternatively, one of the ligands can dissociate prior to nucleophilic attack, a pathway paralleling the S_N1 mechanism of substitution at carbon. In carbon chemistry this results in the intermediate formation of a carbonium ion; analogously a phosphoryl cation would be the primary dissociation product. Both cations demand stabilization; and a special way of stabilization exists in phosphate esters, which have at least one residual free acid function. In this case a proton can be expelled from the acid function with formation of a derivative of metaphosphoric acid - HPO_2 . Monomeric metaphosphoric acid derivatives have been trapped and characterized by F. WESTHEIMER and coworkers (464), but they are highly unstable and tend to form oligomeric or polymeric derivatives, of which the trimetaphosphates are best characterized. Such oligomeric metaphosphates are hypothesized to be intermediates in the transfer of phosphoryl groups derived from phosphoric acid and its monoesters (458). The two alternative mechanisms are shown in schemes 2.2 and 2.3 (see below).

The electron density around the phosphorus atom of phosphoric acid and its esters will be examined next. Three of the four ligands can be either -OH, i.e. acid functions, or ester groups. The fourth is an oxygen atom, and the resulting $P=O$ -bond is polarized by electron withdrawal of the oxygen similar to the $C=O$ -bond in esters of carboxylic or carbonic acids. However, the electron density around the phosphorus atom is not lowered to the same degree as that of carbon due to two facts (53):

1. The phosphorus atom contains empty d-orbitals, which can overlap with p-orbitals of neighbouring oxygen or nitrogen atoms

References, pp. 483-508

containing a lone electron pair. The partial π - π -bonds formed in this manner serve to distribute the positive charge from phosphorus to the neighbouring atoms.

2. In phosphomono- and diesters acid functions remain. The pK_a values for the two acid functions in phosphomonoesters are around 1 and 6, the pK_a of the residual acid function in phosphodiester is between 1 and 2 (25g). Both types of compounds are, therefore, relatively strong acids which fully deprotonate in alkaline media. The resulting negative charge is, of course, "smeared" over the $O=P=O$ -system. The increase in electron density on the P atom and the charge repulsion are responsible for the remarkable stability of phosphate esters to alkaline hydrolysis, which increases in the series phosphotriester < phosphodiester < phosphomonoester (35, 258).

If simple phosphoric acid esters, like ethyl or phenyl, are not very susceptible to nucleophilic attack, what can be done to facilitate the transfer of a phosphoryl group, i.e. to generate a phosphorylating agent? We have to attach an activating group. The structural requirements for such groups have been brilliantly generalized by V. M. CLARK *et al.* (53). According to their basic scheme, all potential phosphorylating agents possess a function described by the general structural formula $P-X-Y-Z$ (X, Y and Z being any element, preferentially C, H, N, O, S, halogen). Z must be (or must be convertible into) a strong electron acceptor, and the X-Y system must be capable of mediating an electron shift from the P-X-bond to Z. Since, in the cases we are looking at, X is mostly oxygen or nitrogen, i.e. atoms containing lone electron pairs, we have to arrange for these electron pairs to be incorporated into a π - π -bond of the X-Y-Z system in order to reduce the stabilization of the P-X-bond by π - π -overlap. This is illustrated in Fig. 2.1. The effect of the X-Y-Z system will be then to produce

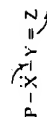
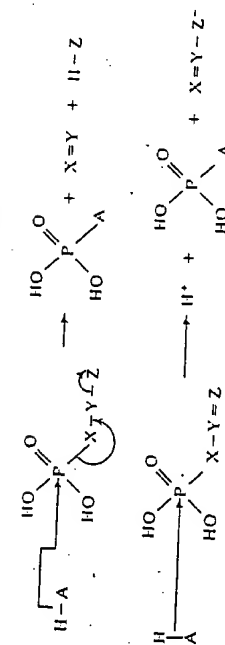
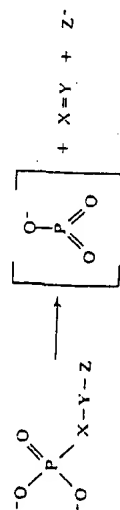


Fig. 2.1

an "energy-rich" bond between P and X, which favors nucleophilic substitution of this group by the result of a negative reaction enthalpy and eventually a positive entropy change due to fragmentation of the X-Y-Z system. Both pathways of decomposition of an activated phosphate, by direct nucleophilic attack or by monomolecular dissociation, are shown in Schemes 2.2 and 2.3.



Scheme 2.2. Decomposition of an activated phosphate by nucleophilic attack on phosphorus



Scheme 2.3. Decomposition of an activated phosphate with formation of metaphosphate

Although this scheme may serve well for designing new potential phosphorylating agents, no prediction is possible as to whether these will be useful in nucleotide chemistry. Activated phosphates, which may easily react with water and ethanol, may be sluggish in phosphorylating the sterically much more hindered nucleosides or oligonucleotides, and only a few of the most powerful activating agents allow the formation of an internucleotide bond. But not only the nature of the attacking nucleophile influences the phosphorylation reaction. The other substituents on the phosphorus atom, and solvents, catalysts and salts, have an effect as well. Generally, as A. M. MICHELSON has pointed out, it is difficult to make a clear distinction between "high-energy" and "low-energy" phosphates in chemical reactions (258). We have already mentioned this difficulty, when discussing protecting groups for the phosphate moiety of nucleotides in Section 1.3.1.

Some phosphorylating agents that have been of aid in the synthesis of nucleotides, oligonucleotides or related biologically active derivatives, are compiled in Table 2.1. The activated phosphates or activating agents are shown in columns 2, 3 and 4 together with the primary activated intermediates they are postulated to produce. Of course, we have to differentiate between two cases: In the first case the phosphorylating agent is a stable activated intermediate, e.g. a nucleoside phosphorochloridate. Then columns 3 and 4 must be identical. In the second case an unstable activated intermediate has to be formed first by reaction of a "low-energy" phosphate, such as a nucleoside phosphate, with a "condensing agent", e.g. dicyclohexyl-

References, pp. 483—508

carbodiimide. Then the structure of the activating agent, which is listed in column 3 of Table 2.1, is different from the structure of the activated intermediate. We will see later in this section, that in the case of dicyclohexylcarbodiimide an imidoylphosphate is assumed to be the primary activated intermediate. The structure of this latter compound is, therefore, shown in column 4. However knowledge of the structure of the primary activated intermediate does not necessarily imply knowledge of the actual pathway of phosphorylation, i.e. whether it follows the mechanism of Scheme 2.2 or the one of Scheme 2.3. This is in most cases unknown, it depends not only on the nature of the activated intermediate, but also on reaction conditions, solvents etc. A thorough discussion of possible reaction paths will be given later for the case of the two best studied condensing agents, dicyclohexylcarbodiimide and sulfonylchlorides.

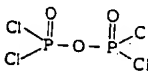
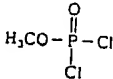
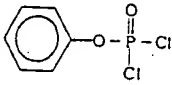
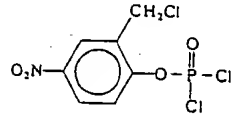
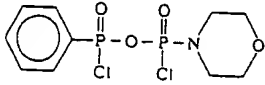
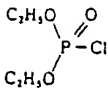
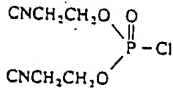
A problem arises from the fact that, depending on what derivative of phosphoric acid we have, we can activate up to 3 functions of the phosphate molecule. Since, in most cases, we wish to form only one phosphate ester linkage at a time, we can solve this problem either by introducing blocking groups into all functions which are not supposed to react or by adopting a route which prevents the participation of other unblocked functions. We will find examples of both strategies in the following discussion of phosphorylating reagents.

The phosphorylating agents in Table 2.1 are grouped into different structural types. The first of these groups includes the phosphoryl halide reagents, i.e. derivatives of POCl_3 . Since POCl_3 itself is a trifunctional acid chloride, care had to be taken to form only one ester linkage. This could be done by using derivatives, which had only one residual acid chloride function and two blocking groups (no. 7—15 in Table 2.1), such as diphenyl- (132, 258) or bis-(β , β ,trichloroethyl-) phosphorochloridate (95). In another line of development multifunctional phosphorochloridates, even POCl_3 itself, could be used avoiding side reactions by a careful selection of reaction conditions and basic catalysts, e.g. 2,6-lutidine (no. 1—6) (216, 350). Nucleotide phosphorofluoridates have been used in the synthesis of oligonucleotides. The chain extension necessitated the use of nucleoside alcohols (see Section 4.3) as nucleophilic partners (440).

Mixed anhydrides have been of great interest in peptide chemistry, and they are so also in oligonucleotide chemistry. In analogy to biological phosphorylations, where derivatives of di- and triphosphoric acid play an important role, similar compounds have been investigated for their use in chemical phosphorylations. These include trimetaphosphate (258, 364, 385), "polyphosphoric acid ester" (prepared from P_2O_5 and stoichiometric amounts of alcohol) (19, 258) and triester-

Table 2.1. Reagents for the Chemical Transfer of Phosphoryl Groups

ences, pp. 483-508

No. Phosphorylating agent	Structural formula	Postulated primary activated intermediate	Application to the synthesis of Nucleotides Nucleotide- Inter- poly- phosphates nucleobonds coenzymes etc.			References
<i>Phosphoryl halides</i>						
1 phosphoryl chloride	POCl_3		+	+	+	(35, 216, 258, 124 sel. 5'-phosphoryl.; 262, 325, 326, 411, 424, 425, 471, 472)
2 pyro-phosphoryl chloride			+	+		(35, 258) sel. 5'-phosphoryl.; (262, 411, 424, 471)
3 methylphosphorodichloridate			+			(415)
4 phenylphosphorodichloridate			+	+	+	(109, 258, 350)
5 2-chloromethyl-4-nitrophenylphosphorodichloridate			+			(134, 135, 282)
6 P ¹ -Phenyl-P ² -morpholino-pyro-phosphorodichloridate			+	+		(162)
7 diethylphosphorochloridate			+			(258)
8 di-β-cyanoethylphosphorochloridate			+			(258)

ences in Nucleotide Synthesis

37

H. Kössel and H. Seiger:

Recent Advances in Polynucleotide Synthesis

Table 2.1 (continued)

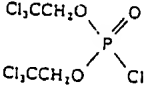
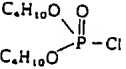
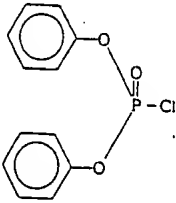
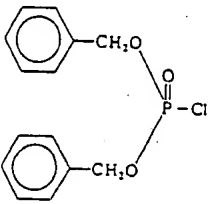
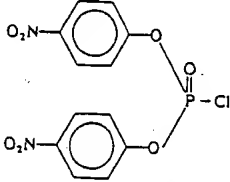
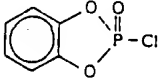
No.	Phosphorylating agent	Structural formula	Postulated primary activated intermediate	Application to the synthesis of nucleotides, nucleotide-polyphosphates, coenzymes etc.	References
9	bis-(β, β -trichloroethyl)-phosphorochloridate			+ sel. 5'-OH	(95)
10	dibutyl-phosphorochloridate			+	(258)
11	diphenyl-phosphorochloridate			+ +	(258)
12	dibenzyl-phosphorochloridate			+ +	(258)
13	bis-(<i>p</i> -nitrophenyl)-phosphorochloridate			+	(132)
14	O-phenylene-phosphorochloridate			+	(191)

Table 2.1 (continued)

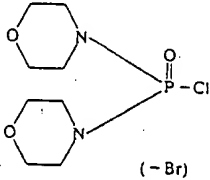

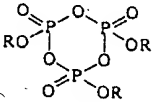
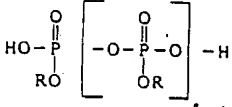
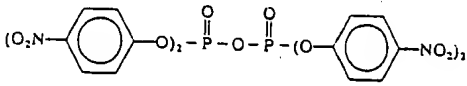
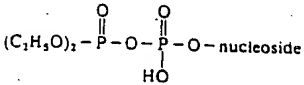
No. Phosphorylating agent	Structural formula	Postulated primary activated intermediate	Application to the synthesis of nucleotides	nucleotide-polyphosphates coenzymes etc.	inter-nucleotide bonds	References
15	di-morpholidic-phosphorochloridate (bromidate)	 (-Br)	+			(258)
15a	nucleoside-phosphorofluoridate		+		+	(426, 440)
<i>mixed anhydrides</i>						
16	trimeta-phosphoric acid and esters	 R = H, Na, Me, Ph	+		+	(166, 258, 458) sel. 2', 3'-OH: (364, 385, 386)
17	poly-phosphoric acid and esters	 R = H, Me, Ph	+		+	(19, 258, 344)
18	tetra- <i>p</i> -nitro-phenyl-pyrophosphate	 (O ₂ N-C ₆ H ₄ -O) ₂ -P-O-P-(O-C ₆ H ₄ -NO ₂) ₂	+			(258)
19	P ⁺ -nucleosidyl-P ²⁺ -diethyl-pyrophosphate	 (C ₂ H ₅ O) ₂ -P-O-P-O-nucleoside	+		+	(61)

Table 2.1 (continued)

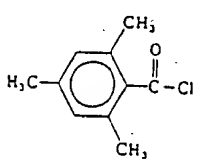
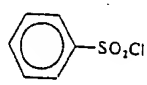
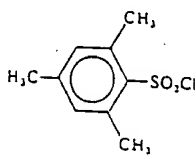
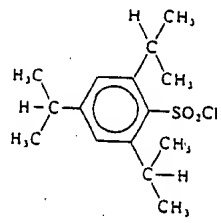
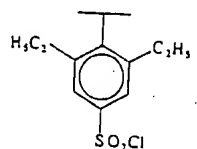
No. Phosphorylating agent	Structural formula	Postulated primary activated intermediate	Application to the synthesis of			References
			nucleotides	nucleotide-polyphosphates	inter-nucleotide bonds	
19a benzyl-, or dibenzyl-, or nucleoside-, phosphoric acid benzoic acid anhydride	$\text{RO}-\text{P}(=\text{O})(\text{OR}')-\text{O}-\text{C}(=\text{O})-\text{C}_6\text{H}_5$ <p>R = benzyl, nucleoside R' = H, benzyl</p>		+	+		(269, 270)
20 mesitylchloride		$-\text{P}(=\text{O})(\text{OR}')-\text{O}-\text{C}(=\text{O})-\text{C}_6\text{H}_2(\text{CH}_3)_3$	+	+		(274)
21 p-toluene-sulfonylchloride		$-\text{P}(=\text{O})(\text{OR}')-\text{O}-\text{SO}_2-\text{C}_6\text{H}_4-\text{CH}_3$	+	+	+	(258)
22 1,3,5-trimethylbenzene-sulfonylchloride (MS)		$-\text{P}(=\text{O})(\text{OR}')-\text{O}-\text{SO}_2-\text{C}_6\text{H}_2(\text{CH}_3)_3$	+	+		(166)
23 1,3,5-triisopropylbenzene-sulfonylchloride (TPS)		$-\text{P}(=\text{O})(\text{OR}')-\text{O}-\text{SO}_2-\text{C}_6\text{H}_2(\text{CH}_2\text{CH}_2\text{CH}_3)_3$	+	+		(248)
24 poly-3,5-diethylstyrene-sulfonylchloride		$-\text{P}(=\text{O})(\text{OR}')-\text{O}-\text{SO}_2-\text{C}_6\text{H}_2(\text{C}_2\text{H}_5)_2$	+	+		(362)

Table 2.1 (continued)

References, pp. 483-508	No. Phosphorylating agent	Structural formula	Postulated primary activated intermediate	Application to the synthesis of			References
				nucleotides	nucleotide-polyphosphates	inter-nucleotide bonds	
	25	trimethylbenzene-sulfonyl-imidazole			+	+	(20)
	25a	trimethylbenzene-sulfonyl-1,2,4-triazolide			+	+	(176a)
	25b	triisopropylbenzene-sulfonyl-1,2,4-triazolide			+	+	(176a)
<i>activated esters</i>							
	26	<i>p</i> -nitrophenyl-phosphate			+	+	(48, 108, 137)
	27	picryl chloride			+	+	(65)
	28	Catechol cyclic phosphate			+		(258)
	29	α -hydroxy-pyridine-phosphates			+	+	(65)

Table 2.1 (continued)

No. Phosphorylating agent	Structural formula	Postulated primary activated intermediate	Application to the synthesis of nucleotides	Application to the synthesis of nucleotide-polyphosphates	Application to the synthesis of inter-nucleotide bonds	References
30 diethyl-(1-ethoxy-2-carbethoxy-vinyl-)phosphate		pyrophosphoric acid triester see 19	+		+	(61)
31 α-bromo-α-cyanoacetamide + triphenylphosphine			+		+	(65, 66)
32 2-methylthio-4H-1,3,2-benzodioxaphosphorin-2-oxide			+			(85)
<i>imidoylphosphates</i>						
33 dicyclohexylcarbodiimide (DCC)			+	+	+	(166, 184, 258, 458)
34 di- <i>p</i> -tolylcarbodiimide		compare no. 33	+			(258)
35 1-cyclohexyl-3-(2N-methylmorpholinoethyl)carbodiimide methosulfate		compare no. 33	+		+	(12, 460)
36 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride		compare no. 33	+		+	(46, 72)

Table 2.1 (continued)

No. Phosphorylating agent	Structural formula	Postulated primary activated intermediate	Application to the synthesis of nucleotides nucleotide- inter- poly- phosphates nucleotide coenzymes bonds etc.			References
37 phosgene + dimethylformamide			+		+	(61, 166)
38 N-ethyl-(methyl)-5-phenylisoxazolium-fluoroborate			+		+	(62, 166)
39 trichloroacetonitrile			+			(65, 258)
<i>activated phosphoramidates</i>						
40 benzyl-hydrogen phosphoramidate			+		+	(35)
41 phosphoromorpholidate			+		+	(184, 252)
42 phosphoryl-imidazole-phosphate + carbonyl-diimidazole			+		+	(59, 246, 258, 324)
43 diimidazolyl-phosphinic acid and derivatives			+			(79, 258)
<i>oxidative phosphorylation</i>						

Application to the synthesis of
nucleotides nucleotide-inter-
poly-
phosphates bonds
coenzymes
etc.

Postulated primary
activated intermediate

Table 2.1 (continued)

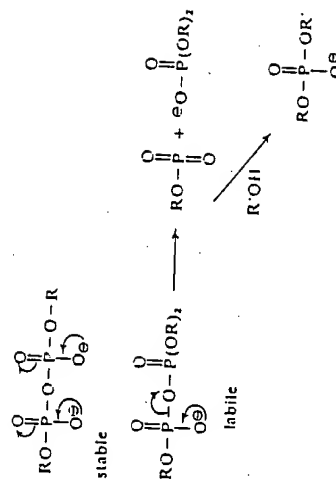
No. Phosphoryl-
Structural formula

ating agent

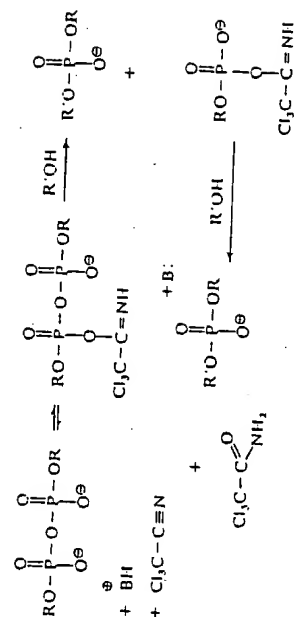
44	naphthohydro- quinone- phosphate + Br ₂			
45	phosphoro-p- hydroxy- anilide + Br ₂			
46	nucleoside-S- ethyl-thio- phosphates + iodine		+	(55.56)
47	triphenyl- phosphine + diphenyl- disulfide		+	(280)

References, pp. 483—508

pyrophosphate (61), accessible *via* a reaction of an enol phosphate with a nucleoside (no. 16—19). F. CRAMER and coworkers have shown in several studies, that in contrast to symmetrical diesters of pyrophosphoric acid, which show a maximum of resonance stabilization, the triesters of pyrophosphoric acid are relatively unstable substances which easily react to transfer the monoester part to a nucleophile (Scheme 2.4) (61).



Scheme 2.4. Comparative reactivity of di- and triesters of pyrophosphoric acid



Scheme 2.5. Activation of a pyrophosphate by trichloroacetonitrile

This mechanism also explains the activation of symmetrically substituted pyrophosphates by condensing agents, such as trichloroacetonitrile (Scheme 2.5). In spite of these interesting mechanistic aspects pyro- and polyphosphate reagents play only a minor role in nucleoside phosphorylations and, especially, in the stepwise synthesis of oligonucleotides.

Among the most widely used phosphorylating agents, however, are mixed anhydrides of phosphoric and sulfonic acids. *p*-Toluenesulfonyl chloride (no. 21) has long been known as a condensing agent, but it

has the disadvantage that it can concurrently tosylate and thus block the alcohol reactant (259). In order to suppress this side reaction, sterically hindered sulfonylchloride reagents, such as mesitylene-sulfonylchloride (abbreviation: MS) (no. 22) (166) and triisopropylbenzenesulfonylchloride (abbreviation: TPS) (no. 23) (248) have been developed by H. G. KRONA and coworkers, and, are, at the moment, the most popular activating agents in polynucleotide chemistry. The mechanism of action of MS and TPS will be discussed later in this section, but it can be said in general, that MS is the "faster", TPS the more selective of the two reagents (248, 274). Recently two new variations have been described, namely trimethylbenzenesulfonylimidazole (no. 25) (20) respectively trimethylbenzene-1,2,4-triazolide (no. 25a) (176a) as an alternative to MS and poly-3,5-diethylstyrene-sulfonylchloride (no. 24) (362) as a polymeric condensing agent (see Section 4.2.4). Mixed anhydrides of carboxylic and phosphoric acids, such as the activated intermediate from mesitylchloride (no. 20) and nucleotides (274) were found less suitable for internucleotide bond formation.

Another prominent group of activating agents, the activated esters (no. 26—31) (48, 61, 65, 258), have been of limited use in nucleotide and internucleotide bond synthesis. This is in contrast to peptide bond formation, where activated esters are of great use as stable and readily available reagents. On the basis of their structure all activated esters belong or can be related to a class of compounds called enol phosphates. Syntheses and reactions of enol phosphates, have been reviewed earlier by F. W. LICHTENTHALER (245). Enol phosphates possess an activated ester residue because they contain a "quasi" preformed aldehyde or ketone which can easily be liberated as an excellent leaving group, when for instance a reagent such as a proton induces the electron shift shown in Fig. 2.6.

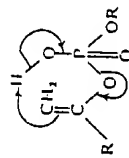


Fig. 2.6

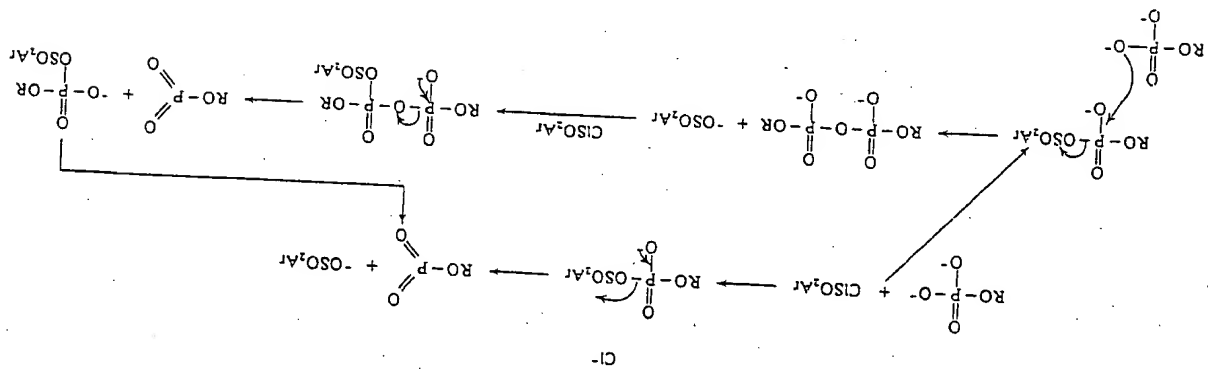
A slight modification of the enol phosphate concept leads us to an extremely useful class of activating agents, the imidoylphosphate intermediates. Imidoylphosphates are analogous to enol phosphates, but the Y-Z system is a C=N double bond instead of a C=C double bond. The C=N bond is much more easily protonated, e.g. by pyridinium

ions in a water-free pyridine medium. Protonation produces a very labile species which easily fragments with liberation of metaphosphate.

The most important of these reagents are the carbodiimides (no. 33—36), among which the dicyclohexylcarbodiimide (no. 33) (144, 258, 274) in particular has been a standard condensing agent in nucleotide as well as peptide chemistry for many years. Compared to the sulfonylchloride reagents discussed above, dicyclohexylcarbodiimide (abbreviation: DCC) has two disadvantages: in order to obtain good yields one needs significantly longer reaction times and the by-product, dicyclohexylurea, is difficult to remove, since it is only slightly soluble in a few solvents. However, the carbodiimides are not very sensitive to water around neutral pH, so it was possible to construct and successfully use water-soluble carbodiimides, e.g. no. 35 and 36 of Table 2.1, for condensations in aqueous media (46, 72, 460). Other condensing agents, designed to give imidoyl phosphate intermediates (no. 37—39) (61, 62, 65, 166) have not been as successful in the stepwise synthesis of oligonucleotides, however, reagents such as trichloroacetoneitrile (no. 39) (65, 258) and also picryl chloride (no. 27) (65) have shown good results in the polycondensation of nucleotides.

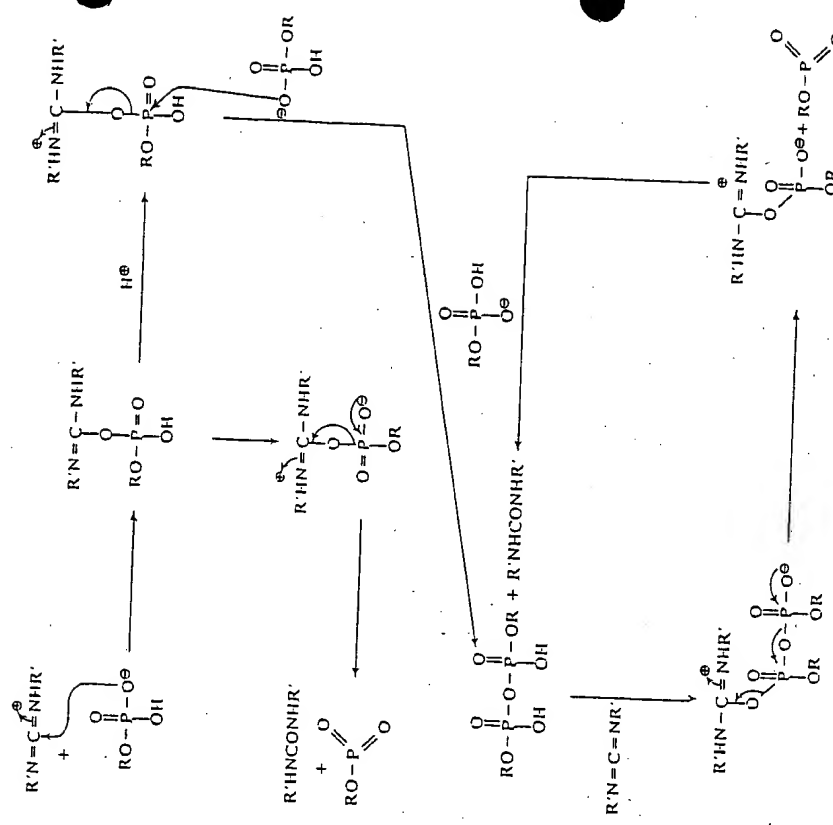
Whereas all activations we have discussed so far proceed with P-O-cleavage, a P-N bond is broken in the nucleophilic displacement of activated phosphoramidates (no. 40—43) (6, 35, 184, 258). Although the activation of these compounds proceeds by protonation similar to the imidoylphosphates, they are not as "energy-rich" as these latter derivatives, because the basicity of the nitrogen atom is reduced by π - π -overlap to the phosphorus atom as discussed earlier. The main range of application of the activated phosphoramidates, especially the phosphoromorpholides, lies in the synthesis of nucleotide coenzymes.

A final group of compounds for phosphoryl transfer consists of reagents useful for oxidative phosphorylation. Oxidative phosphorylation is one of the basic phosphorylation processes of biological systems, and thus quinol phosphates have also been tested for their ability to mediate chemical phosphorylations. Although interesting from a mechanistic standpoint, these studies have not yielded any phosphorylating agent of major importance. In the case of the activating residues no. 44 and 45, the quinol phosphates were activated by bromine oxidation (35, 316). Through oxidation of the *p*-hydroxyanilinate residue (no. 45) a moderate yield of dTpdT could be obtained; thus this residue could be used as an activatable blocking group (see Section 1.4) (316). However, the need for bromine oxidation precludes a more general use of this method. Another example of an activation during blocking group cleavage is the oxidative removal of the S-ethyl-phosphorothioate residue with iodine (no. 46; see also Section 1.4 and Scheme 1.6) (55).



Scheme 2.7. Possible mechanisms for formation of a metaphosphate with sulfonyl chlorides

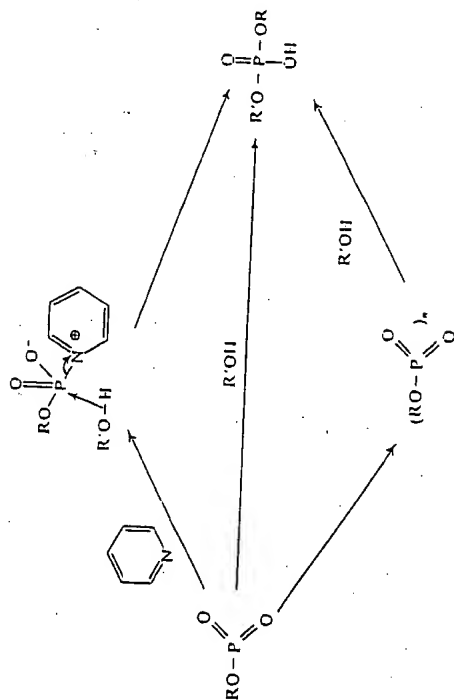
A similar "activated" phosphorothioate intermediate seems to be present in phosphorylations with 2-methylthio-4H-1,3,2-benzodioxaphosphorin-oxide (no. 32) (85), although in this case an ester rearrangement rather than a redox reaction produces the activated species. Another recent addition to the spectrum of phosphorylating agents is triphenylphosphine, which, in the presence of dipyridyldisulfide as oxidising agent, can form an intermediate activated phosphonium phosphate (no. 47) (280). Triphenylphosphine as activating agent for alcohols will be discussed in the following section.



Scheme 2.8. Possible pathways for formation of a metaphosphate with dicyclohexylcarbodiimide

In concluding this section we will take a closer look at the mechanism of action of the two most frequently used phosphorylating agents, namely sulfonylchlorides and dicyclohexylcarbodiimide. Reaction schemes have been advanced by different authors (35, 166, 248, 258), the picture which emerges is, however, still relatively complex. Most certainly, more than one mechanistic pathway is possible. For the sulfonyl chlorides some mechanistic alternatives are shown in Scheme 2.7 (166, 258). One could be termed a "direct" route: A phosphomonoester is activated by sulfonylation and subsequent decomposition of the mixed anhydride to give metaphosphate. Alternatively metaphosphate could be formed by an "indirect" route via a symmetrical pyrophosphate, which can again be activated by sulfonylchlorides, as discussed earlier.

The same two alternatives are shown in Scheme 2.8 for dicyclohexylcarbodiimide activation. Once the monomeric metaphosphate is formed, it will most likely be trapped by the solvent pyridine to give a phosphoryl-pyridinium ion. This pyridinium complex (or the metaphosphate itself) could then react with all other nucleophiles present, namely metaphosphate to give oligo- or polyphosphate intermediates, the phosphoric acid starting compound to revert back to pyrophosphate or, finally an alcohol resp. nucleoside to give a phosphodiester linkage (Scheme 2.9).



Scheme 2.9. Possible pathways for conversion of a metaphosphate to a phosphodiester

A decision as to which of these different routes is preferred is still difficult. Recent studies by G. M. BLACKBURN and coworkers (22, 23, 24) showed, that no phosphodiester formation would occur, when the

References, pp. 483-503

phosphoniumoester component was linked to a polymer support, thus preventing the formation of di- or polyphosphate intermediates. On the other hand, excellent yields of phosphotriester were obtained by R. L. LETSINGER and coworkers, when support-bound phosphodiester (blocked nucleoside-monophosphates) were activated, in the presence of a nucleoside, with TPS (not, however, with DCC) (230, 238). Obviously, different mechanistic routes prevail with different phosphorylating agents as well as with different reactants.

2.2. Transfer of a Phosphate Group

Phosphate transfer to nucleosides or nucleotides has been observed to occur on activation of nucleoside hydroxyl groups. The phosphate transfer to activated hydroxyl groups proceeds by a nucleophilic attack of phosphate or nucleotide on the carbon atom next to the hydroxyl group which is displaced, i.e. in most cases on C3'. The mechanism can be formulated as an S_N2 -type, as shown in Fig. 2.10. Activation of the

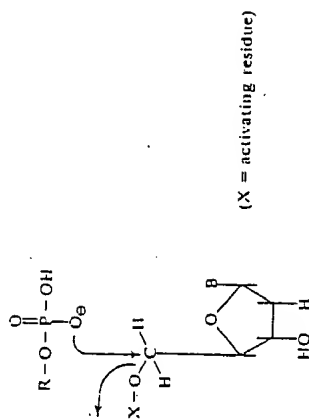
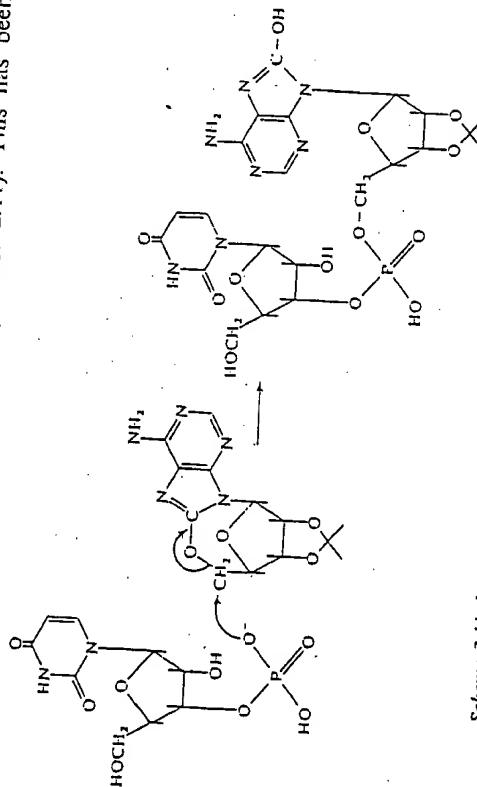


Fig. 2.10

hydroxyl group must significantly lower the electron density on the carbon atom in question, thus facilitating nucleophilic attack, and must also transform the alcohol group into a good leaving group. This has been achieved by O. MITSUNOBU and coworkers (263) by activation with triphenylphosphine and azodicarboxylate, a triphenylphosphonium derivative of the nucleoside being the presumed intermediate. E. W. HAEFFNER (122) has reported, alcohol activation as the result of mesylation which results in nucleoside phosphorylation and even, in small yield, in the formation of the dinucleoside phosphate dTpdT; however, this could be done only at elevated temperature. A similar

example from the older literature is the phosphorylation of 3'-O-acetyl-5'-iodo-5'-deoxythymidine with silver dibenzyl phosphate (258). Along similar lines, the synthesis of dinucleoside phosphates from 5'-chloro-5'-deoxynucleoside and tri-n-butylammonium-3'-uridyate was reported more recently (429).

A series of investigations has dealt with anionic attack of phosphates and nucleotides on cyclic anhydro nucleosides. The formation of an ether or sulfide bridge between C3' or C3' and C2' or C8' of a pyrimidine resp. purine base makes these carbon atoms of the sugar ring susceptible to nucleophilic attack (Scheme 2.11). This has been



Scheme 2.11. Internucleotide bond formation with anhydro nucleosides

used for the formation of several dinucleoside phosphates (1, 269, 270, 283, 427, 428, 478). Generally the yields are moderate even at higher temperatures. To obtain a good yield of rCprA from O², C^{3'}-anhydro-cyclocytidine and 5'-AMP, the nucleotide was additionally activated as a phosphoric acid - benzoic acid anhydride (269, 270). Of course, the formation of natural 3'-5'-internucleotide linkages implies that the reaction proceeds with complete inversion at C3', and this was not completely the case.

2.3. Miscellaneous Chemical Phosphorylation Reactions

This section describes several approaches, which are either mechanistically completely different from the ones reviewed in Sections 2.1 and 2.2 or not yet well enough understood to allow an unequivocal classification.

References, pp. 483-508

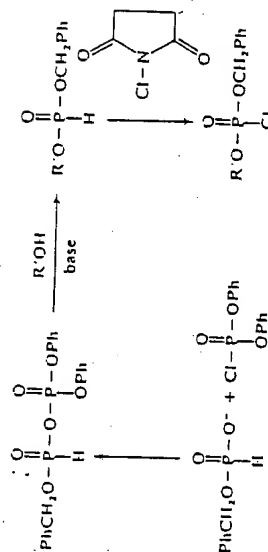
The phosphorylation methods mainly to be dealt with are

1. phosphorylations involving oxidation of an intermediate phosphorous acid ester of a nucleoside,
2. so-called "thermal" phosphorylations, and
3. phosphorylations under possible prebiotic conditions, involving "prebiotic phosphorylating agents", mineral surfaces etc.

Different reasons have led to these investigations. The oxidation of nucleoside phosphites has since long been known as a good method for the introduction of the phosphate moiety into nucleosides which are not sensitive to oxidising agents; however, there seems to be no significant advantage as compared with more straightforward methods for introducing the phosphoryl moiety such as the use of DCC or TPS. The direct "thermal" conversion of nucleosides into nucleotides by inorganic phosphate with no need for protection or activation could be an approach unsurpassed in its simplicity; however, as long as a mixture of isomers results and the yields of a single product cannot be significantly improved, the main value lies in a one-step synthesis of labelled nucleotides. Phosphorylations on mineral surfaces, finally, have been studied mainly with regard to solving questions about the prebiotic formation of nucleotides. None of these three methods is as yet of any importance in oligonucleotide synthesis.

2.3.1. Phosphorylation by Oxidation of Nucleoside Phosphites

The phosphorylation of alcohols and amines by a mixture of tetra-chloromethane and phosphorous acid dialkyl esters was described as early as 1945 by Lord Todd and coworkers. It was demonstrated that a diesterphosphorochloridate is formed as intermediate (35, 258). Nucleoside phosphorylations were similarly done with O-benzylphosphorous-O-diphenylphosphoric anhydride, a reagent prepared from



Scheme 2.12. Preparation of an alkyl-benzyl-phosphorochloridate

diphenylphosphorochloridate and monobenzyl phosphite (35). Alcohols react with this reagent by nucleophilic attack at the less acidic component of the anhydride to give the alkyl benzyl phosphite, which is then chlorinated with N-chlorosuccinimide to the alkyl benzyl phosphorochloridate. This can either be hydrolyzed to the corresponding phosphate or used directly as an activated nucleotide derivative (Scheme 2.12). In other cases the phosphite residue was introduced by reaction with phosphorus trichloride (469, 473), phosphorous acid + DCC (35) or trichloromethane phosphonic acid dialkylester (158).

2.3.2. "Thermal" Phosphorylation

Since 1965 several investigations have been published, in which inorganic phosphate was transferred to nucleosides without introduction of activating groups into any of the reaction partners. Since this approach has been named "thermal" phosphorylation (164, 275, 276, 277, 278, 345). Typically, a nucleoside and phosphoric acid (often applied as the tri-n-butylammonium salt) are heated at reflux in dry dimethylformamide for several hours, the resulting generally complex mixture of products being separated according to charge. Other sources of phosphate can be used, such as pyrophosphoric acid or the nucleotides themselves. In an example of the latter case, studied by T. UEDA and I. KAWAI (452), 5'-AMP was refluxed in DMF to give predominantly adenosine and adenosine-2',3'-cyclic phosphate.

A thermal phosphorylation resulting in the conversion of nucleotides into a homologous mixture of oligo- and polynucleotides has been studied by O. PONGS and P. O. P. TS'O (339, 340). The reaction is done in refluxing dimethylformamide with catalysis by β -imidazolyl-4(5)-propionic acid, triethylamine hydrochloride or other proton donors. The polycondensations were achieved with unblocked nucleotides. In contrast to earlier experiments of H. SCHRAMM and coworkers (258) on the polyphosphate-catalyzed polycondensation of unblocked nucleotides the products in this case were shown to contain nearly 95% of 3'-5'-phosphodiester linkages. Unfortunately, neither the yields nor the stereochemical purity of the product are as yet sufficiently high to make this very simple approach preparatively workable.

All these cases of "thermal" introduction or migration of phosphate residues have been attributed to intermediate activation of phosphate by dimethylformamide, but an activated pyrophosphate could also be a plausible intermediate (see Section 2.1) (452). Thus, although the overall reaction would suggest phosphate attack, the moiety which really is transferred seems to be a phosphoryl group. However, as the

References, pp. 483-508

mechanistic aspects await further clarification, we have preferred to describe this approach in the present section. It should be added, that this reaction need not necessarily be a thermal one, since it has been shown that the reaction of inorganic phosphate with nucleosides proceeds very well at room temperature with predominant formation of 5'-nucleotides, if formamide is used as reaction medium (333).

2.3.3. Prebiotic Phosphorylations

For several years an increasing number of studies has been devoted to shedding light on the manner in which nucleic acid components could have been formed under primitive earth conditions. Of course, the considerations governing work of this type are completely different from those valid for preparative organic chemistry. The main question is: "Is it possible that all the assumed reaction partner might have been present in reasonable quantity and close contact in a primitive earth environment." Yields of products, on the other hand, are not as important as in preparative reactions, since the time at the disposal of nature to accumulate a certain product is incomparably longer than the observation time of a laboratory. Since this review is concerned primarily with preparative aspects of polynucleotide chemistry, it may suffice to retrace two lines of development in the field of prebiotic phosphorylation.

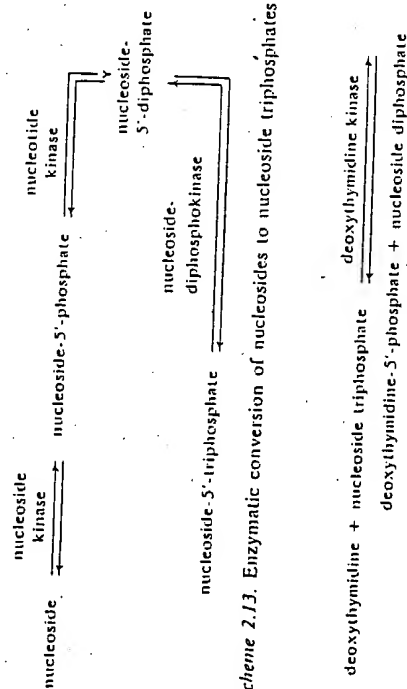
Most probably inorganic phosphates must have been the source of phosphoric acid and nucleosides or sugars the phosphate acceptors in such reactions. The working hypotheses differ in answers to the question how phosphorylation could have been mediated. One line of work is based on the assumption, that primitive oceans could have contained "prebiotic" phosphorylating agents such as dicyanogen, malonitrile or acrylonitrile, all of which have been demonstrated to allow the formation of nucleotides or sugar phosphates from nucleosides and D-ribose (126, 367). A second line of development has attempted to demonstrate that nucleotides could have been formed in contact with mineral surfaces. The mineral surface may act merely as a catalyst, but especially high yields of nucleotides have been obtained when the mineral present was a phosphate donor, such as hydroxylapatite, and when urea and ammonium chloride were added as adjuvants (250, 302, 323).

2.4. Enzymic Phosphorylation

Kinases and phosphorylases are biological catalysts for the introduction and transfer of a phosphate moiety. Kinases catalyze the

phosphate addition to a biological intermediate, phosphorylates the phosphorylytic cleavage of such compounds.

The conversion of nucleosides to nucleotides catalyzed by nucleoside kinases was found to be an intermediate step in the metabolic pathway leading from nucleosides to nucleoside triphosphates according to Scheme 2.13 (33). The overall reaction, for the example of thymidine



Scheme 2.13. Enzymatic conversion of nucleosides to nucleoside triphosphates

Scheme 2.14

kinase, is shown in Scheme 2.14. Kinases are known for several of the common nucleosides and three species, namely adenosine- (204), thymidine- (320, 321) and deoxycytidine (273) kinase have been purified from *E. coli*, calf thymus and other sources. Substrate specificity was relatively high in the case of adenosine- and thymidine kinase, whereas deoxycytidine kinase could phosphorylate also ara-cytidine, deoxyadenosine and deoxyguanosine. Nucleoside triphosphates act as phosphate donors, whereby mostly the end product of the metabolic chain, i.e. the respective triphosphate of the preferred substrate, is a strong inhibitor. The products are specifically 5'-nucleotides.

A "low-energy" phosphate transfer has been demonstrated for plant and animal tissues by E. CHARGAFF (36), who recently succeeded in purification of an enzyme from *E. coli*, which transfers phosphate from low-energy organic donors to nucleosides, nucleoside-5'-phosphates and deoxynucleoside-5'-triphosphates. With the exception of adenosine, the nucleosides are converted almost exclusively into 2'- and 3'-nucleotides. Thymidine and its derivatives are the best acceptors of phosphate groups. The fact that nucleoside-2'(3')-phosphates are the main products suggests a regulatory role for this enzyme rather than an

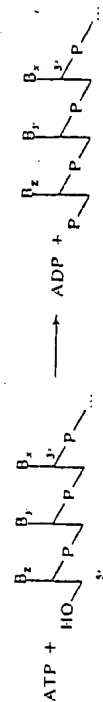
References, pp. 483-508

involvement in the biosynthesis of nucleoside polyphosphates. A phosphotransferase from carrot was similarly used to prepare a variety of 5'-nucleotides and analogs for DNA polymerase binding studies (129). A low-energy phosphate transfer catalyzed by cells of *Pseudomonas mesophilus* has been described as a preparative approach to guanylic and inosinic acid in a Japanese patent (267).

Enzyme preparations from cells of *B. ammoniagenes* have been used in several studies, especially in the synthesis of labelled nucleotides (93, 284, 285, 301). The enzyme catalyzes the transfer of a phosphoribosyl group to nucleobases. Uracil is converted nearly quantitatively into 5'-uridylic acid. Phosphoribosylation of adenine takes place with lower efficiency, whereas guanine and cytosine are practically not phosphoribosylated at all. Labelled orotidine-5'-phosphate was also prepared.

A different approach to enzymatic phosphorylation of nucleosides has been described by A. HOLY and G. KOWOLIK (156). The authors reacted nucleosides with guanosine-2',3'-cyclic phosphate under catalysis by T₁ RNase (compare Section 5.3). Subsequent cleavage of the internucleotide linkage by snake venom phosphodiesterase transfers the phosphate residue to the 5'-position of the starting nucleoside. Although the yields did not exceed 30%, the method may be interesting as an extremely mild procedure for the phosphorylation of very labile nucleoside analogs. Also this method, like other enzymatic phosphorylations of nucleosides, has the general advantage, that specific substitutions can be obtained with unblocked starting compounds.

The enzyme polynucleotide kinase, which transfers orthophosphate from ATP to polynucleotides, oligonucleotides and even nucleoside-3'-phosphates, has been isolated from T4-bacteriophage infected *E. coli* by C. C. RICHARDSON (357). The overall reaction, as shown in Scheme 2.15, is a specific 5'-phosphorylation of the oligo- or poly-

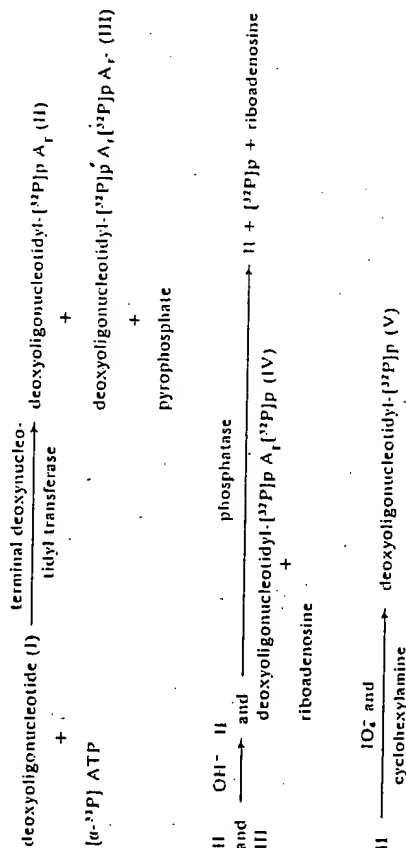


Scheme 2.15

nucleotide. Ribo-mono- and polynucleotides were equal as substrates to deoxy compounds. Mg^{++} and 2-mercaptoethanol are required. As an analytical tool this enzyme is of value in the specific labelling and analysis of the 5'-terminus of polynucleotides and in testing the specificity of exonucleases. It has been of even greater interest in the chemical synthesis of polynucleotides, since it allows the phosphorylation

of oligonucleotide fragments, which can then be joined by polynucleotide ligase (see Section 4.1.1) (6, 198). The *in vivo* role of this enzyme is not quite clear. Since it does not accept nucleosides its action is different from that of the above-mentioned nucleoside kinases. The possibility of an *in vivo* production and condensation of activated oligonucleotide fragments has been suggested.

Complementary to the use of polynucleotide kinase is a method described by H. KÖSEK and R. ROYCHOUDHURY (208) for the specific addition of phosphate to the 3'-end of an oligodeoxynucleotide chain. This is done by the sequence of enzymatic and chemical reactions shown in Scheme 2.16. This method is especially useful for attaching a radio-



Scheme 2.16. Phosphorylation method of Kössel and Roychoudhury

actively-labelled phosphate to the 3'-terminus. By spleen phosphodiesterase digestion the label is transferred to the 3'-terminal unit of the sequence, thus allowing an end group determination of polynucleotides.

Enzymes which catalyze phosphorolytic cleavage of nucleosides, nucleotides or polynucleotides, such as nucleoside phosphorylases or polynucleotide phosphorylase, are of great value as analytical tools and/or, as instruments for the synthesis of oligo- and polynucleotides. These aspects will be treated in detail in Section 5. Since, for example, the displacement of an internucleotidic linkage by inorganic phosphate is not a preparative approach to nucleoside polyphosphates, we can abstain from discussing these enzymes in this section.

3. Separation Techniques

Purification and characterization of products are major time consuming steps in most, if not all, of the conventional synthetic procedures. The possible reduction of these steps to the products of the final reaction has been one main motivation for the efforts already invested in polymer support synthesis. At the same time, however, several new separation techniques applicable to conventional synthetic procedures could be developed for large scale preparations as well as for work on an analytical scale. In addition to the introduction of well-established absorbents as, for instance, Sephadex or Biogel for polynucleotide mixtures, improvements could also be achieved by the development of new adsorbent types specific for certain functional groups of protected or unprotected oligonucleotides.

Furthermore, progress could be made by introduction of new solvent systems for elution or for chromatography, or by new combinations of already known solvent systems. Finally entirely new lipophilic protecting groups have been devised which allow the specific extraction of intermediates whereby time consuming column steps can be avoided or simplified.

3.1. Column Procedures

3.1.1. Column Chromatography on Conventional Adsorbent Types

Application of Sephadex column chromatography for the preparative separation of oligonucleotide mixtures has been reported in several

studies (5, 39, 129, 286, 287, 288, 315, 318, 347, 400, 433, 454). This method appears attractive as the desired synthetic products — usually those of the highest chain lengths within a reaction mixture — will be eluted within the exclusion volume, whereby rapid isolation within a comparatively small elution volume is guaranteed. A further advantage consists in the low buffer concentration (usually triethylammonium bicarbonate) of the eluent which simplifies further work-up of the isolated compounds. Although successful separations of products resulting from single nucleotide additions have been reported (287, 288, 315), a prerequisite to satisfactory separation seems to be the condition that the compounds to be separated differ maximally from one to another in size (288, 347). Even if this is achieved by adjusting the synthetic plan so that approximate doubling of the chain lengths occurs during any of the reaction steps, additional complications may arise from other factors. Thus, with protected nucleotides retardation has been observed to increase in the following order: $pT < dpbzA < dpanC < dpibuG$ (347), and this order is reflected in the elution patterns of derived oligomers. Conformational influences on the elution behaviour of oligonucleotides during Sephadex column chromatography have been documented by the successful separation of 2'—5' dinucleoside monophosphates from their 3'—5' isomers (433). The observation that certain nucleotide derivatives — notably d-panC and its relatives are eluted in two peaks may also reflect conformational influence (347). In spite of all these possible complications, the gel permeation technique at least in selected cases seems to compare favourably with the more time-consuming DEAE-cellulose column chromatography of protected oligonucleotides and several cases have been reported where the products isolated by Sephadex column chromatography were sufficiently pure for further condensation steps (287, 288, 315). Gel permeation techniques on Sephadex or Biogel have found especially widespread application for the separation of unprotected oligo- (144, 221, 433) and polynucleotides (2, 39, 129, 130, 195, 399, 400, 402, 446, 454). Particularly suitable for this technique seem to be product mixtures resulting from polynucleotide ligase catalyzed reactions as on the one hand conventional DEAE cellulose column chromatography does not provide the necessary resolution power for chain lengths in the range of 20 and more nucleotides and as on the other hand the prerequisite of a relatively large difference in the chain lengths between the fragments to be coupled and the products is always fulfilled.

DEAE-cellulose column chromatography in spite of its drawbacks is still the technique most widely used for the separation of protected oligonucleotides (5, 37, 38, 40, 218a, 219, 315, 341, 457). As the absorption is largely governed by ionic forces, the elution order of a

References, pp. 433—503

polymeric mixture primarily reflects the number of negative charges of the various components. This therefore results essentially in separation according to chain length. Complications however, arise from an additional retardation order ($T < dpbzA < dpanC < dpibuG$) which is obviously due to nonionic interaction and which is reflected also in the elution patterns of derived oligomers. A similar interaction seems to be even stronger with compounds containing the highly lipophilic monomethoxytrityl group at the 5'-end. While ethanol gradients superimposed on the salt gradients can be applied in order to selectively retard monomethoxytrityl containing products (37, 40, 205, 457), use of salt gradients containing the more hydrophilic methanol effect complete absorption of monomethoxytrityl protected components even in the presence of high salt. Based on this observation a simple technique using two successive salt gradients with methanol and ethanol on the same column was devised for the separation of 5'-monomethoxytrityl containing oligomers (in the deoxy series usually the starting block and the desired product) from the remaining products of a block condensation mixture (379, 380).

One major drawback of DEAE cellulose consists in its reduced resolution power for protected oligomers of higher chain length. In this range (ten nucleotides and longer) it is therefore necessary to increase the chain length by several nucleotide units at a time (block condensation) in order to obtain satisfactory resolution. On the other hand, analytical DEAE-cellulose columns in the presence of 7 M urea, have proven to be useful tools for the characterization or final purification of unprotected oligomers in the chain length range of 8 to 20, especially as the standard paper chromatographic procedures also are severely limited in this size range. As evidenced by its routine use in recent work on the synthesis of the tRNA-Ala-Gene (37, 38, 40, 218a, 219, 315, 457), the disadvantage of this time-consuming column technique which requires in addition the complete deprotection of the oligomers to be characterized, seems to be fully counterbalanced by its high resolution power. While in most cases neutral salt gradients were applied, use of acidic ammonium formate (pH 3.5) seems also to be possible even in the case of purine-containing deoxyoligonucleotides (37).

A promising technique for the rapid analytical separation of unprotected or fully protected oligonucleotide mixtures appears to be high pressure liquid chromatography on a pellicular weak anion exchanger consisting of a polymeric aliphatic amine (131). The time necessary for separation of one optical density unit of a condensation mixture was reported to be less than 30 minutes.

3.1.2. Column Chromatography on Newly Developed Adsorbent Types

The nonionic interaction between the highly lipophilic 5'-O-protecting monomethoxytrityl group and cellulose is strongly increased if the cellulose matrix itself is modified by naphthoylation (4, 5) or by tritylation (6, 41). *Tritylated cellulose* seems to be an especially powerful tool for the selective adsorption of monomethoxytrityl-containing compounds (in the deoxy series usually the starting block and the desired product). After elution of the nontritylated components of a given condensation mixture in the presence of low alcohol concentration, elution of the trityl-containing components is effected simply by a switch to higher alcohol concentration in the eluent. More recently this separation principle has been extended to other lipophilic 5'-O-protecting groups such as the 2-S-naphthylmercaptoethyl group (7). The fact that only low salt concentrations are necessary in the eluents simplifies further workup of the compounds isolated by this procedure. The elegance of this technique appears, however, to be counterbalanced to some extent by the requirement for further fractionation of the monomethoxytrityl-containing compound mixtures which are usually composed of the unreacted starting block and of the desired product. A general scheme for selective blocking of the 3'-hydroxyl group of unreacted starting material by naphthylisocarbamoyl has been proposed which would allow further condensation steps at the 3'-hydroxyl group of the desired products exclusively (8); as a consequence further fractionation of the mixtures containing the tritylated oligonucleotide products could be avoided.

Increased affinity of oligonucleotide compounds containing aromatic protecting groups is also observed with *benzoylated DEAE-Sephadex* (259, 289, 290, 290a). Specific aromatic 5'-phosphate protecting groups such as benzhydrylamidyl or 2-phenylmercaptoethyl cause adsorption of the respective nucleotide derivatives in the absence of alcohol even at high salt concentrations. After elution of the components free of aromatic 5'-phosphate protecting groups in the presence of aqueous salt gradients, isolation of the 5'-phosphate protected derivatives is achieved by addition of 50% ethanol to the eluent. Maintenance of high salt concentrations is, however, also necessary for effective elution. As in the case of trityl cellulose, this ethanolic fraction (containing the desired product and one of the unutilized condensation components) has to be further fractionated, and separation of the two main components (and other minor side products) could be achieved by Sephadex column chromatography (290a). In view of the two column steps necessary for effective purification of the final product, the usefulness of this technique seems limited.

References, pp. 483-508

Cellulose and polymethacrylic acid gels, to which dihydroxyboryl groups had been attached covalently, have been described as column chromatographic adsorbents, specific for the *cis*-diol group of a variety of ribonucleotide derivatives (377, 381, 460). Thus, due to complex formation of the dihydroxyboryl groups of the respective matrices with the *cis*-diol group of ribonucleoside residues at slightly alkaline pH, specific adsorption of ribonucleosides, of 5'-ribonucleotides, of ribooligonucleotides lacking a 3'-phosphate group, of 3'-ribonucleoside terminated oligodeoxynucleotides and of free tRNA is observed, whereas deoxynucleosides, deoxynucleotides, deoxyligonucleotides, ribooligonucleotides with a 3'-terminal phosphate and aminoacylated tRNA are eluted within the void volume. The adsorbed compounds can subsequently be recovered from the column matrix by lowering the pH of the eluent buffer to neutrality. This technique has proved especially useful for the preparative separation of mixtures containing the component (pT)_n and (pT)_n-pU, where the lack of differences in size or in net charge does not allow separation by any of the other conventional techniques (381).

3.2. Extraction Procedures

The use of extraction procedures in the chemical synthesis of protected dinucleoside monophosphates from a 5'-trityl protected nucleoside and a protected nucleoside 5'-phosphate has been reported earlier (205). After extraction of the unreacted nucleoside derivative by ethyl acetate, the protected dinucleoside monophosphate can be separated from the unreacted mononucleotide derivative by extraction with chloroform. The extractability of the protected dinucleoside monophosphate seems to depend on the presence of the lipophilic 5'-O-trityl protecting group and on the presence of lipophilic counterions of the product such as pyridinium or triethylammonium cations. More recently this extraction principle could successfully be extended to the separation of a protected trinucleoside diphosphate and even of a protected pentanucleoside tetraphosphate (38, 40, 315). In order to exploit extraction procedures also for the isolation of protected mono- and dinucleotides containing 5'-terminal phosphate groups lipophilic protecting groups containing one or more aromatic rings have been developed for the protection of 5'-phosphomonoester groups (3, 7, 176). Thus, synthesis and organic solvent extraction of all 16 possible deoxydinucleotides in the protected form has been reported after *p*-aminophenyltrityl-methane was used for the amidation of the respective 5'-terminal phosphate groups (3). Introduction of the 2-S-phenylmercaptoethyl or

of the 2-S-naphthylmercaptoethyl group for the protection of 5'-terminal phosphates seems also to allow selective solvent extraction of protected mononucleotide derivatives (7).

There seems to be no doubt that extraction procedures do simplify the total workup of condensation reactions in many cases and that therefore, whenever possible, extraction procedures should be used in order to avoid the more time-consuming column separation steps. Care is, however, necessary to make sure that products isolated by extraction procedures are pure enough for subsequent condensation steps. In addition, due to the detergent effect of protected oligonucleotides, it is sometimes difficult to separate the two solvent layers after thorough mixing and the resulting emulsions are occasionally stable enough to resist even prolonged centrifugation at maximum speed.

3.3. Miscellaneous Techniques

The use of thin layer chromatography for the separation of oligonucleotides has been reviewed recently (348). In the meantime a few additional systems for the analytical separation of oligomeric mixtures have been published (3, 5, 37, 123, 219, 223, 260, 291, 293, 318, 457). In the triester approach (see below) extensive use has been made of preparative as well as analytical thin layer chromatography (42, 76, 78, 236, 300, 350, 418, 419) and of short column chromatography (73). The latter technique seems especially useful for the rapid separation and purification of large quantities of protected oligonucleotides.

Synthetic homooligomers from the series d(pT)_n, d(pA)_n and d(pC)_n and a number of oligomers of varying base composition have been characterized by analytical ultracentrifugation (257). Though this method would in principle allow also separation of oligomers from each other, its application seems to be useful mainly for the chain length characterization of oligomers.

4. Formation of Internucleotide Linkages by Chemical Synthesis

4.1. Conventional Methods

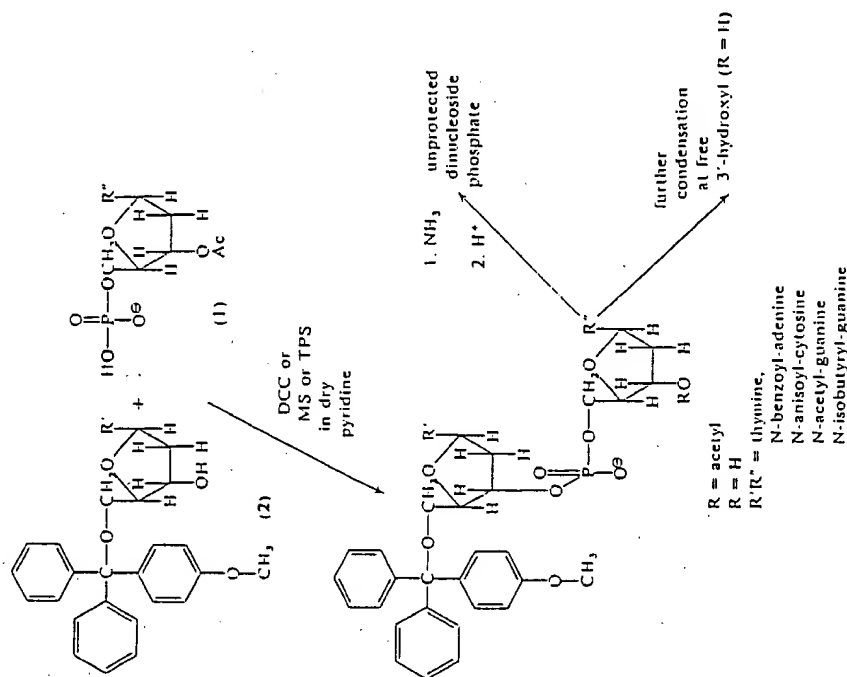
4.1.1. Synthesis in the Deoxy Series

4.1.1.1. Synthesis via Phosphodiester Intermediates

During the past ten years chemical synthesis of deoxyribopolynucleotides up to a chain length of twenty nucleotide units has become

References, pp. 483-508

feasible owing to the pioneering work of KUDORANA and co-workers (185, 186, 187, 188, 319). The crucial steps, the formation of the phosphodiester linkages, are achieved by successive condensations (1; Scheme 4.1) with the monoester group of nucleotidic components (2; Scheme 4.1) with the 3'-hydroxyl of the respective nucleosidic components (2; Scheme 4.1). While dicyclohexylcarbodiimide (DCC) has been used as condensing agent in most of the earlier work, its application has now become limited more or less to the preparation of protected dinucleoside monophosphates, as in contrast to the aromatic sulfonyl chlorides no anionic products are introduced by DCC during the course of the



Scheme 4.1. Formation of internucleotide linkages in the deoxy series via phosphodiester intermediates

reaction (monovalent anions such as chloride and arylsulfonates produced by hydrolysis of aromatic sulfonylchlorides would not be separable from protected dinucleoside monophosphates by conventional DEAE cellulose column chromatography as the latter also possess one negative net charge). Although DCC in principle seems an effective condensing agent for the preparation of longer chains (207) also, there are major drawbacks because of the required longer reaction times and the necessity for complete absence of even traces of strongly basic amines in the reaction mixtures. Therefore preferential use has been made of aromatic sulfonyl chlorides such as mesitylene sulfonyl chloride (MS) and trisopropyl sulfonyl chloride (TPS) as condensing agents for the synthesis of higher oligonucleotides (166, 248).

Production of relatively large amounts of the monoanions chloride and arylsulfonate is the only possible problem arising from the use of aromatic sulfonyl chlorides as condensing agents. In most cases, however, separation of the nucleotide products from these monoanions is readily achieved by preparative DEAE cellulose column chromatography and even in the case of protected dinucleoside monophosphates where separation on DEAE cellulose would not be possible, extraction procedures can be applied (see above).

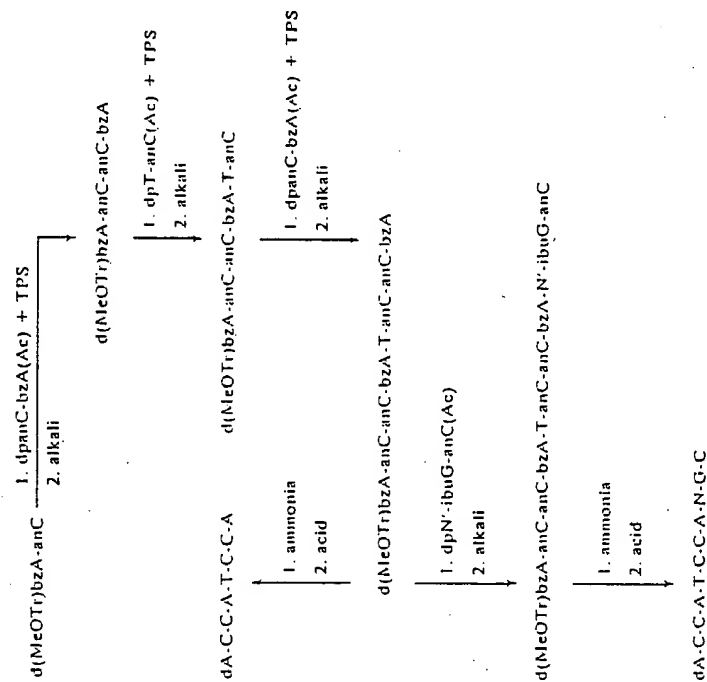
The solvent routinely used for chemical condensation reactions is dry pyridine. This solvent combines suitable volatility (for ready removal by evaporation under mild conditions) with solvent power generally satisfactory for even longer chains of protected oligonucleotides. The use of other solvents such as lutidine and hexamethylphosphotriamide has been reported (207) but no apparent advantage over the more volatile pyridine was observed.

In most of the work classical acyl protecting groups for the amino functions of the base residues have been used (anisoyl for cytosine, benzoyl for adenine, acetyl or isobutyryl for guanine) for both condensation components (185, 186, 187, 188).

Protection by acetylation of the 3'-hydroxyl group of the nucleotidic component (1, Scheme 4.1) is necessary to prevent self-condensation of the latter. The 5'-hydroxyl group of the nucleosidic component (2, Scheme 4.1) is protected by the acid-labile monomethoxytrityl residue. If the 3'-hydroxyl-containing component itself carries a phosphomonoester group at its 5'-end, protection against self-condensation is also necessary. This can be achieved by esterification with excess of β -cyanoethanol; the resulting β -cyanoethyl group can be removed selectively after each condensation step by strong alkali treatment. More recently a variety of other protecting groups for the masking of terminal phosphate residues has been introduced to permit selective removal without cleavage of the N-acyl groups which protect the bases. From

References, pp. 483-508

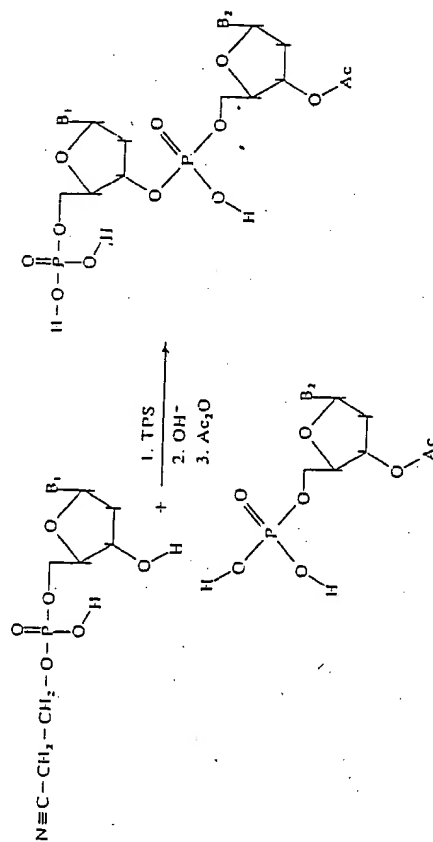
this, two general routes have been developed for the synthesis of longer oligonucleotide chains in the deoxy-series: the first approach, outlined in Scheme 4.2, for the synthesis of an undecamer (379) consists in repetitive condensation steps at the 3'-end of a growing chain containing a protected 5'-hydroxyl group. This approach, already developed earlier for polynucleotide synthesis in relation to the genetic code (186, 187), has been used extensively in more recent work dealing with the synthesis of various gene segments including two complete sets corresponding to tRNA genes (Table 4.1). Virtually throughout all this work the acid labile monomethoxytrityl group has been used for the 5'-hydroxyl protection. While the 5'-end of the growing chain remains blocked by this group throughout the entire reaction sequence (except for the very last deprotection step), the 3'-terminal O-acetyl groups, still present at the extended chains immediately after each condensation step, are selectively removed by alkali treatment before every subsequent condensation step is carried



Scheme 4.2. Synthesis of the octanucleotide dA-C-C-A-T-C-C-A and of the undecanucleotides dA-C-C-A-T-C-C-A-N-G-C

out. Thus, an overall growth direction from the left (5'-end) to the right (3'-end) is the result. After final deprotection by ammonia and acid treatment products without 5'-terminal phosphate residues are isolated.

In contrast to this, the second general route leads to the synthesis of 5'-terminal phosphate containing nucleotide chains. As outlined in Scheme 4.3 protection of the 5'-terminal phosphate group present in the 3'-hydroxyl bearing components is then necessary. In many cases



B₁, B₂ = T, A, G, C, G^{ac}, G^{ibu}

Scheme 4.3. Synthesis of 5'-terminal phosphate containing nucleotide chains in the deoxy series

the cyanoethyl group has served as phosphate protecting group in such reactions and the preparation of "blocks" by reactions analogous to the one outlined in Scheme 4.3 has become almost routine (see references in Table 4.1). A major disadvantage of the cyanoethyl (and of other alkali-labile) phosphate protecting group is that it is removed by the alkali treatment necessary for the hydrolysis of the 3'-terminal O-acetyl groups after each condensation step. Thus, cyanoethylation of the 5'-terminal phosphate residues has to be carried out before every subsequent reaction step. In order to eliminate this complication, a number of alkali stable phosphate protecting groups has been developed more recently, which (like the 5'-O-trityl group) can remain at the 5'-phosphate group throughout the entire reaction sequences necessary to build up the longer nucleotide chains (see foregoing chapter on protecting groups). This approach seems to be useful not only for the preparation of oligonucleotide blocks (3, 7, 55, 56, 176, 290a, 312), as the synthesis of comparatively long chains has also been

References, pp. 483-508

reported. Thus, by using the phosphothioethyl group three dodecanucleotides and one tridecanucleotide of specific sequences corresponding to fragments of a DNA coding for a derivative of S-peptide of ribonuclease A could be synthesized (58, 129, 146, 341) in addition to a dodecanucleotide sequence constituting the 5'-terminus of the r-strand of λ -phage DNA (145) (see Table 4.1). In analogous reaction sequences but using the phenylmercaptoethyl group for the protection of the 5'-terminal phosphate, the synthesis of two deoxyribopolynucleotide fragments of chain lengths nine and twelve has been reported which contain a natural sequence of the phage T4 lysozyme gene (291) (see Table 4.1).

Table 4.1. Oligodeoxynucleotides of Specific Sequence Synthesized Chemically by the Phosphodiester Method*

No.	Sequence	Resp. Gene	References
1	T-G-G-T-G-G-A-C-G-A-G-I	tRNA ^{Ala} _{yeast}	(315)
2	P-C-C-A-C-C-A	tRNA ^{Ala} _{yeast}	(315)
3	C-C-G-G-A-C-T-C-G-I	tRNA ^{Ala} _{yeast}	(219a)
4	C-C-G-G-A-A-T-C	tRNA ^{Ala} _{yeast}	(219a)
5	C-C-G-G-T-T-C-G-A-T-I	tRNA ^{Ala} _{yeast}	(219a)
6	G-A-A-C-C-G-G-A-G-A-C-T-C-T-C-C-C-A-T-G	tRNA ^{Ala} _{yeast}	(457)
7	P-A-G-A-G-T-C-I	tRNA ^{Ala} _{yeast}	(219)
8	G-C-T-C-C-T-T-T-A-G-C-A-T-G-G-G-A-G-A-G	tRNA ^{Ala} _{yeast}	(37)
9	C-T-A-A-G	tRNA ^{Ala} _{yeast}	(219)
10	G-G-A-G-C-G-C-G-C-I	tRNA ^{Ala} _{yeast}	(5)
11	T-C-G-G-T-A-G-C-G-C	tRNA ^{Ala} _{yeast}	(40)
12	A-C-C-G-A-C-T-A-C-G	tRNA ^{Ala} _{yeast}	(40)
13	T-G-G-C-G-C-G-T-A-G	tRNA ^{Ala} _{yeast}	(40)
14	C-G-C-C-A-C-A-C-G-C-C-C	tRNA ^{Ala} _{yeast}	(38)
15	G-G-G-C-G-T-G	tRNA ^{Ala} _{yeast}	(38)
16	C-T-A-C-C-G-A-C-T-A-C-G	tRNA ^{Ala} _{yeast}	(5)
17	C-T-A-A-G-G-G-A-G	tRNA ^{Ala} _{yeast}	(219)
18	T-C-T-C-C-G-G-T-I	tRNA ^{Ala} _{yeast}	(219)
19	C-G-A-G	tRNA ^{Ala} _{yeast}	(190)

* The blocks used are indicated by underlining of the respective partial sequences. As chain growth occurs always from left to right, the sequences of the intermediates can also be deduced. Thus, for the synthesis of 1, the oligonucleotides TG, TGG, TGGT, TGGTG, TGGTGG, TGGTGGG, and TGGTGGGACG in the protected form are intermediates whereas for the synthesis of 2 the intermediates pCC and pCCA in the protected form are derived.

Table 4.1 (continued)

No.	Sequence	Resp. Gene	References
20	C-C-C-A-C-C-A-C-C-A	IRNA ^{SV, SU} _{E. coli}	(188, 190)
21	T-C-G-A-A-T-C-C-T-T-C	IRNA ^{SV, SU} _{E. coli}	(188, 190)
22	T-G-G-G-G-A-A-G-G-A	IRNA ^{SV, SU} _{E. coli}	(188, 190)
23	T-T-C-G-A-A-C-C-T	IRNA ^{SV, SU} _{E. coli}	(188, 190)
24	T-T-C-G-A-A-G-G-T	IRNA ^{SV, SU} _{E. coli}	(188, 190)
25	C-G-T-C-A-T-C-G-A-C	IRNA ^{SV, SU} _{E. coli}	(188, 190)
26	C-T-A-A-A-T-C-T-G-C	IRNA ^{SV, SU} _{E. coli}	(188, 190)
27	T-C-G-A-A-G-T-C-G-A	IRNA ^{SV, SU} _{E. coli}	(188, 190)
28	T-G-A-C-G-G-C-A-G-A	IRNA ^{SV, SU} _{E. coli}	(188, 190)
29	T-T-A-G-A-G-T-C-T	IRNA ^{SV, SU} _{E. coli}	(188, 190)
30	G-C-T-C-C-C-T-T-T-G	IRNA ^{SV, SU} _{E. coli}	(188, 190)
31	G-C-C-G-C-T-C-G-G-G-A	IRNA ^{SV, SU} _{E. coli}	(188, 190)
32	C-C-C-A-C-C-A-C-G-G	IRNA ^{SV, SU} _{E. coli}	(188, 190)
33	G-A-G-C-A-G-A-C-T	IRNA ^{SV, SU} _{E. coli}	(190)
34	C-G-G-C-C-A-A-G-G	IRNA ^{SV, SU} _{E. coli}	(188, 190)
35	G-G-G-T-T-C-C-G-G-A-G	IRNA ^{SV, SU} _{E. coli}	(188, 190)
36	G-G-T-G-G-G-G-T-T-C-C	IRNA ^{SV, SU} _{E. coli}	(188, 190)
37	A-T-T-A-C-C-C-G-T	IRNA ^{SV, SU} _{E. coli}	(190)
38	A-G-T-A-A-A-G-C	IRNA ^{SV, SU} _{E. coli}	(190)
39	G-G-A-G-C-A-G-G-C-C	IRNA ^{SV, SU} _{E. coli}	(190)
40	G-C-T-T-C-C-C-G-A-T-A-A-G	IRNA ^{SV, SU} _{E. coli}	(190)
41	G-T-A-A-T-G-C-T-T-T	IRNA ^{SV, SU} _{E. coli}	(190)
42	T-A-C-T-G-G-C-C-T	IRNA ^{SV, SU} _{E. coli}	(190)
43	G-C-T-C-C-C-T-T-A-T-C-G	IRNA ^{SV, SU} _{E. coli}	(190)
44	G-G-A-A-G-C	IRNA ^{SV, SU} _{E. coli}	(190)
45	p T-T-A-A-T-T-A-C-A-A-T-A	Bovine insulin chain A	(288)
46	p A-T-T-T-T-C-C-A-A-T-T-G	Bovine insulin chain A	(288)
47	p A-T-A-C-A-A-A-C-T-A-C-A	Bovine insulin chain A	(288)
48	p A-T-T-A-A-G-T-G-A-T-G-G	T ₄ -Lysozyme	(291)
49	p A-C-T-T-T-T-T-G-T	T ₄ -Lysozyme	(291)
50	p A-A-G-A-C-A-G-C-A-T-A-T	Pancreatic RNase A (S-peptide)	(341)

References, pp. 483-508

Recent Advances in Polynucleotide Synthesis

51	p T-T-A-A-T-C-C-A-T-A-T-G-C	Pancreatic RNase A (S-peptide)	(38)
52	p T-G-C-T-A-A-A-T-T-T-G-A	Pancreatic RNase A (S-peptide)	(146)
53	p A-A-A-T-T-T-G-A-A-A	Pancreatic RNase A (S-peptide)	(146)
54	p T-G-T-C-T-T-T-C-A-A-A-T	Pancreatic RNase A (S-peptide)	(129)
55	p T-T-A-G-C-A-G-C-C-G-C-A-G	Pancreatic RNase A (S-peptide)	(131)
56	p A-G-G-T-C-G-C-C-G-C-C-C	Sticky end of phage λ	(145)
57	A-C-C-A-T-C-C-A-A-G-C	Coat protein, (379) phage fd.	
58	A-C-C-A-T-C-C-A-C-G-C	Coat protein, (379) phage fd.	
59	A-C-C-A-T-C-C-A-G-G-C	Coat protein, (379) phage fd.	
60	A-C-C-A-T-C-C-A-T-G-C	Coat protein, (379) phage fd.	
61	A-C-C-A-T-T-C-A-A-G-C	Coat protein, (380) phage fd.	
62	A-C-C-A-T-T-C-A-C-G-C	Coat protein, (380) phage fd.	
63	A-C-C-A-T-T-C-A-G-G-C	Coat protein, (380) phage fd.	
64	A-C-C-A-T-T-C-A-T-G-C	Coat protein, (380) phage fd.	
65	A-G-A-A-T-A-A-A-A	Ribosomal binding site of phage φX174	(383)
66	C-A-G-T-T-T-G-A-G-C-A-T	Endolysine of phage λ	(467)
67	A-G-T-C-C-A-T-C-A-C-T-T	T ₄ -Lysozyme (467a)	
68	A-G-T-C-C-A-T-C-A-C-T-T-A-A	T ₄ -Lysozyme (467a)	
69	p C-C-A-A-A-C-C-A-A-A	T ₄ -Lysozyme (467a)	
70	G-T-T-C-T-G	—	(20)
71	p G-G-T-T-T-C-G-T-G-G	—	(20)

Polycondensation reactions. If mononucleotides protected only on the base residues are reacted with a condensing agent, polycondensation to a homologous series of polynucleotides occurs (Scheme 4.4). During



Scheme 4.4

synthetic work related to the genetic code (186, 187) this technique has been extended to the polymerization of preformed di-, tri-, and tetranucleotide blocks (Scheme 4.5; 286, 308). By this technique



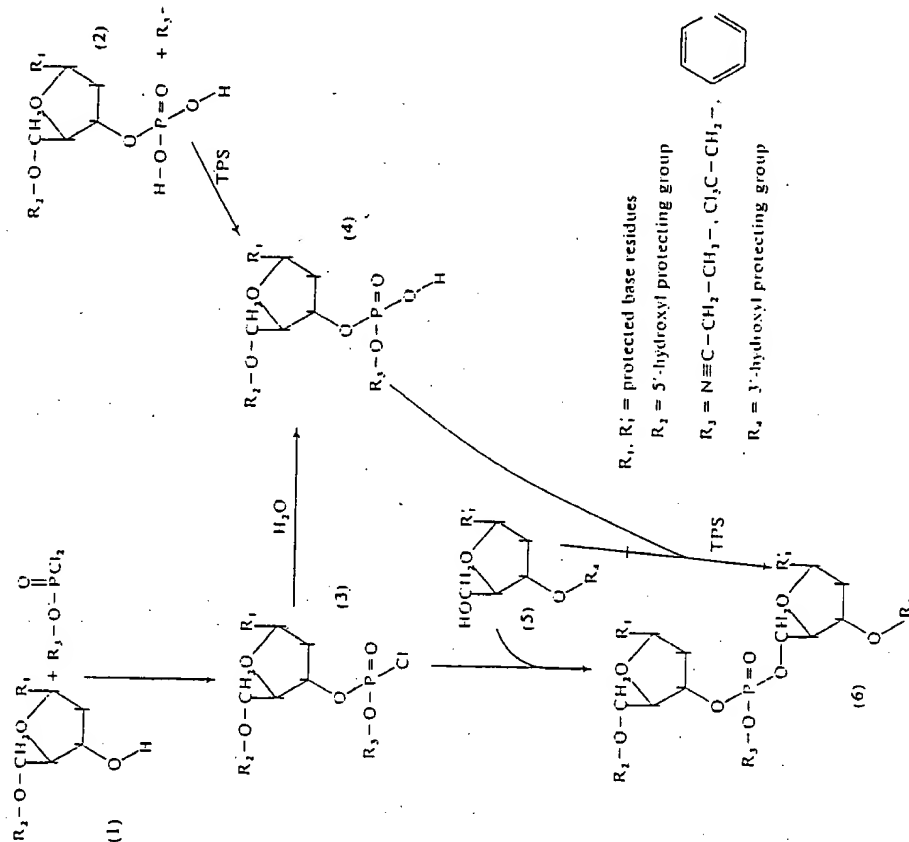
Scheme 4.5

complete oligonucleotide series including members of comparatively large size are readily accessible within short time. The method, however, is generally limited to the synthesis of polynucleotides containing repeating nucleotide sequences (in most cases homopolymers).

More recently polycondensation has been carried out with a mixture of 5'-thymidylic acid and 2'-3'-O-dibenzoyl uridine-5'-phosphate which after ammonia treatment leads to a mixture of the series (pT)_n and (pT)_npU, (381). As the components can be separated by a combination of conventional DEAE cellulose column chromatography and chromatography on a borate-containing matrix (359, 381), this approach seems generally applicable for the synthesis of 3'-ribonucleoside terminated homooligodeoxynucleotides. Recently polycondensation techniques have also been utilized for the synthesis of copolymers of specific sequence (396, 398a, 398b; see also section 1.4.2).

A characteristic feature of the condensation methods described in this chapter is that the phosphodiester functions, already present in the reaction components or formed during the course of the reaction, are left unprotected. This approach, generally called *diester approach*, introduces two main disadvantages: first, dialkyl phosphate anions are not chemically inert to the conditions necessary for synthesis and therefore side reactions such as pyrophosphate formation or cleavage of internucleotide bonds by pyridine can occur (166). These – and perhaps other – side reactions seem to be increasingly severe in the synthesis of longer chains as a substantial decrease in the general yields has quite regularly been encountered in the preparations of higher oligonucleo-

tides. Secondly, the partially protected intermediates are insoluble in organic solvents (with the exception of protected dinucleoside monophosphates and other shorter oligonucleotides carrying highly lipophilic protecting groups, see the section on protecting groups and on extraction methods) and it is therefore necessary to use laborious fractionation procedures such as DEAE cellulose chromatography to purify them after each condensation step. It seemed likely that both these disadvantages would be overcome if the internucleotide linkages of the intermediates



Scheme 4.6. Formation of the internucleotide linkage in the deoxy series via phosphotriester intermediates

were protected by further esterification to phosphotriesters and a number of studies have been reported on this so-called *triesther approach* to be reviewed in the following chapter.

4.1.1.2. Synthesis *via* Phosphotriester Intermediates

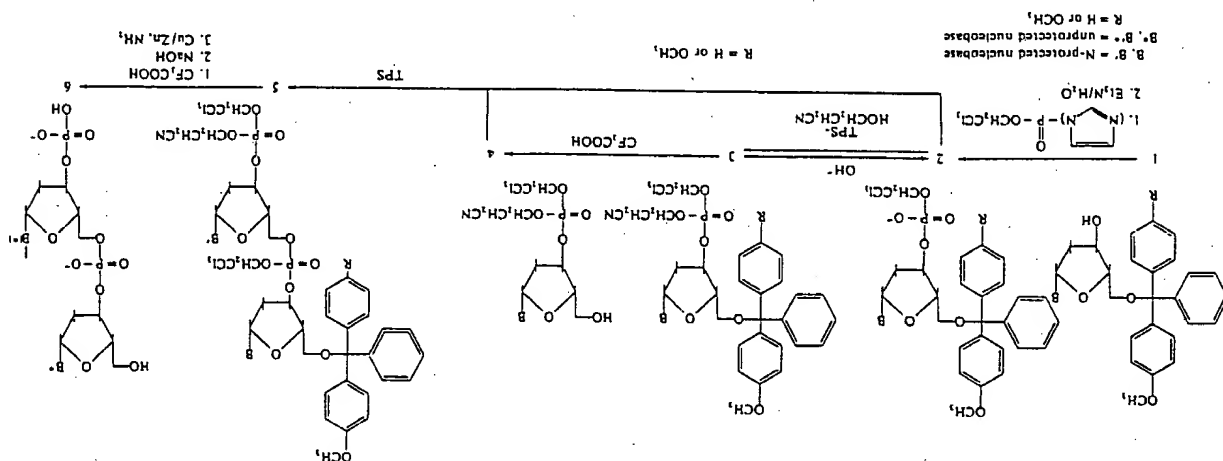
In the deoxy series protection of internucleotidic phosphate by β -cyanoethyl (87, 235, 236), trichlorethyl (42, 75, 76, 78, 94, 418), phenyl (73, 352, 358), and substituted phenyl groups (352, 358) has been reported. As outlined in Scheme 4.6 the key intermediate (4) is produced either by direct esterification of the monophosphate containing component (2) or by concomitant introduction of the protecting group with the phosphate residue. In the latter case a suitably protected 3'-hydroxy component (1) is first reacted with β -cyanoethyl dihydrogen phosphate (87, 235) or with phenyl dihydrogen phosphate (73) in the presence of a condensing agent as TPS (Scheme 4.6). In other cases the 3'-terminal diester residue is introduced by reaction of a suitably protected 3'-hydroxyl component (1) with trichlorethyl or phenyl phosphorodichloridate (75, 78, 352). The resulting 3'-phosphorochloridate derivatives (3) can be used directly as activated phosphate components for the reaction with a suitably protected 5'-hydroxyl derivative (5); alternatively the chloride is removed by hydrolysis, whereby the desired 3'-terminal diester derivatives (4) are obtained. The internucleotidic linkage finally is formed by condensation of the latter with suitably protected 5'-hydroxyl bearing components (5) in the presence of TPS as condensing agent. Approaches in which esterifications in all steps are achieved by a condensing agent [pathway (2) \rightarrow (4) \rightarrow (6) of Scheme 4.6] seem to be more favourable than earlier approaches in which the respective aryl or alkyl phosphorodichloridates were used in stoichiometric amount for successive reactions with one 3'-hydroxyl and one 5'-hydroxyl component [Scheme 4.6; pathway (1) \rightarrow (3) \rightarrow (6)].

In order to allow repeated condensation steps for the synthesis of longer chains, protecting groups at the 3'- and 5'-termini have to be selected such that selective removal after each condensation step is possible.

Thus, when alkali-labile protecting groups are used at the one end, acid labile protecting groups have to be applied at the other end (42, 73, 75, 76, 78, 236, 352). Following this principle oligothymidylic acids could be synthesized by the stepwise (42, 76, 78, 236, 352) or blockwise (42, 73, 75, 78, 236) approach up to a chain length of eight by using components with protected hydroxyl functions at the respective 3'- and 5'-termini. While this approach has been used for the stepwise synthesis of dinucleoside monophosphates and trinucleoside diphosphate

References, pp. 483--508

Scheme 4.7. Formation of the internucleotide linkage in the deoxy series via phosphorintermediates



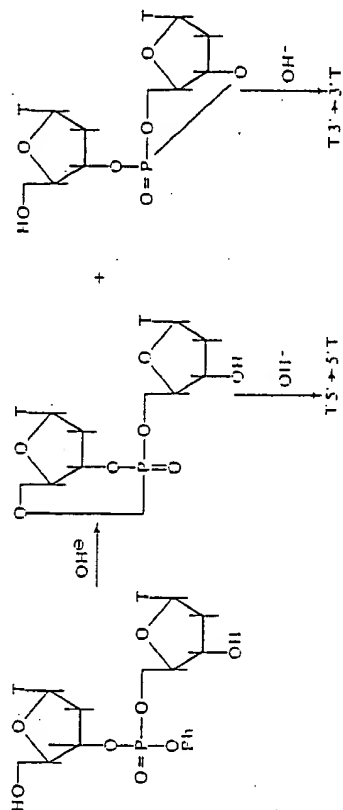
containing also base residues other than thymine (78, 236), a more recent approach in addition seems to allow block condensation with all four standard nucleotide derivatives (42). This is based on condensation of a nucleoside, 3'-phosphate trichlorethyl ester, which carries an acid labile blocking function on the 5'-hydroxyl, with the free 5'-hydroxyl group of a second nucleoside 3'-phosphate trichlorethyl ester, in which the phosphate carries an additional base-labile group (Scheme 4.7). The resulting fully protected dinucleotides can then selectively be de-blocked at either the 5'- or the 3'-terminus by the use of acid or base and the resulting partially protected dinucleotides can be used in further condensation reactions. The masked phosphate can be used in further group offers the advantage of permitting the introduction of the phosphate at the mononucleotide stage, rather than before each subsequent condensation. Using this approach a variety of di-, tri-, and tetranucleotides containing all the four common bases could be synthesized in good yields. The tetranucleotides were prepared by block condensations from two dinucleotide units. In view of these encouraging results it seems desirable to extend this technique to the synthesis of longer chains containing specific sequences. Whether in this case no severe limitations arise from the acid sensitivity of the purine glycoside linkages (especially of d bzA residues containing oligonucleotides) or from other side reactions (see below) has yet to be tested.

A systematic study on the polycondensation using triesterintermediates has been reported in the oligothymidylic acid series (87). When 5'-O-monomethoxytrityl-thymidine 3'-[(β-cyanoethyl)phosphate] and thymidine 3'-[(β-cyanoethyl)phosphate] were reacted with aromatic sulfonyl chlorides for 12–14 days a mixture of oligothymidylic acids, the largest being the pentanucleotide, was obtained. It was demonstrated that the failure to yield longer chains is mainly due to the formation of C-pyridinium-thymidine nucleotides, a side reaction previously also observed in the diester approach (166). Owing to the longer reaction times necessary for the triester reactions this side reactions seem to be more severe in the triester approach. The formation of the C-pyridinium nucleotides could be avoided by using collidine as solvent. Production of longer oligonucleotide chains was, however, not observed either when collidine was used (87).

A careful study of the synthesis of the dTpT *via* the triester approach has shown that deprotection of the triester group by alkali treatment leads to isomerization of the internucleotide linkage to 5'–5' and 3'–3'-derivatives if the 5'-terminal and/or 3'-terminal hydroxyl functions are free during the alkali treatment (282a, 358). Cyclic triesters seem to be the intermediates of this isomerization (Scheme 4.8) as evidenced from the fact that no isomerization is observed when the hydroxyl func-

References, pp. 483–508

tions are blocked by alkali resistant groups (358). Protection of the hydroxyl functions, for instance by tetrahydropyranylation (73), is therefore necessary before removal of the phosphate protecting aryl groups of oligonucleotides can be carried out by alkali treatment.



Scheme 4.8. Isomerization during alkali treatment of triesters

In view of the progress reviewed here and in view of the reported synthesis of a nonanucleotide in the riboseries (see below) by the triester approach, it seems not unlikely that it finally will allow synthesis of specific gene segments sufficiently long to permit joining reactions which are catalyzed by polynucleotide ligase. The triester approach seems particularly promising as the expectation that rapid and effective purification of intermediates could be effected by silica gel column chromatography (232), by preparative thin layer chromatography (see separation techniques) or by short column chromatography (see separation techniques) on a comparatively large scale was fulfilled. As, on the other hand, the products obtained by the triester approach, sometimes do contain considerable amounts of side products (73, 87) – even in the oligothymidylic acid series – purification by conventional DEAE cellulose chromatography seems to be necessary, at least for the final products of longer reaction sequences. In the light of the side reactions encountered during both approaches, the question remains open which method – the diester or the triester approach – is superior to the other as far as minimizing such products is concerned.

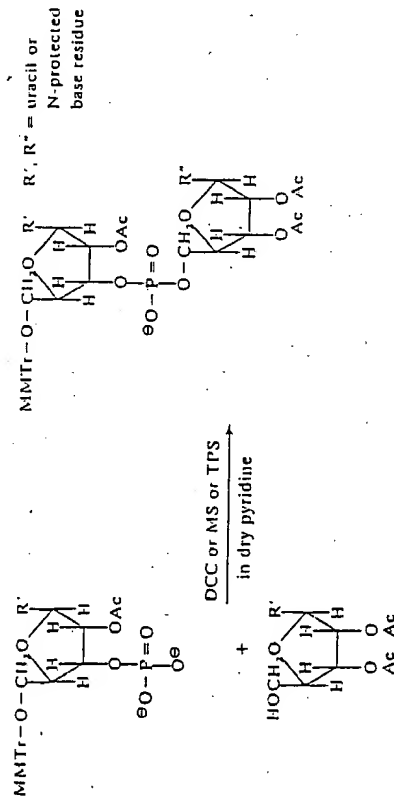
4.1.2. Synthesis in the Ribo Series

Chemical synthesis of ribopolynucleotides is complicated by the presence of 2'-hydroxyl groups, for which special protecting groups had to be introduced (see chapter of protecting groups). Since the 3'-

phosphomonoesters are more readily available and as the existence of a 3'-phosphate group facilitates the selective protection of the 2'-hydroxyl function of 5'-trityl-N-acyl derivatives, the principle of condensing a protected 3'-phosphate with the free 5'-hydroxyl group of a protected nucleoside component has been used in both the diester and the triester approach (Schemes 4.9 and 4.11). As regards condensing agents and protecting groups for the common functional groups, profound differences do not exist between conventional synthetic methods in both the ribo and deoxy series. In the ribo series, too, the diester approach developed earlier has been complemented by the more recently introduced triester approach.

4.1.2.1. Synthesis via Phosphodiester Intermediates

In the classical diester approach utilized, for instance, for the synthesis of all 64 possible ribotrinucleoside diphosphates (249), a suitably protected 3'-phosphate containing the acid labile 5'-monomethoxytrityl protecting group is condensed with the free 5'-hydroxyl group of a protected nucleoside (Scheme 4.9). After selective deblocking of the 5'-hydroxyl function of the resulting protected dinucleoside phosphate, further condensation can be carried out with a new protected 3'-phosphate leading to a protected trinucleoside diphosphate.

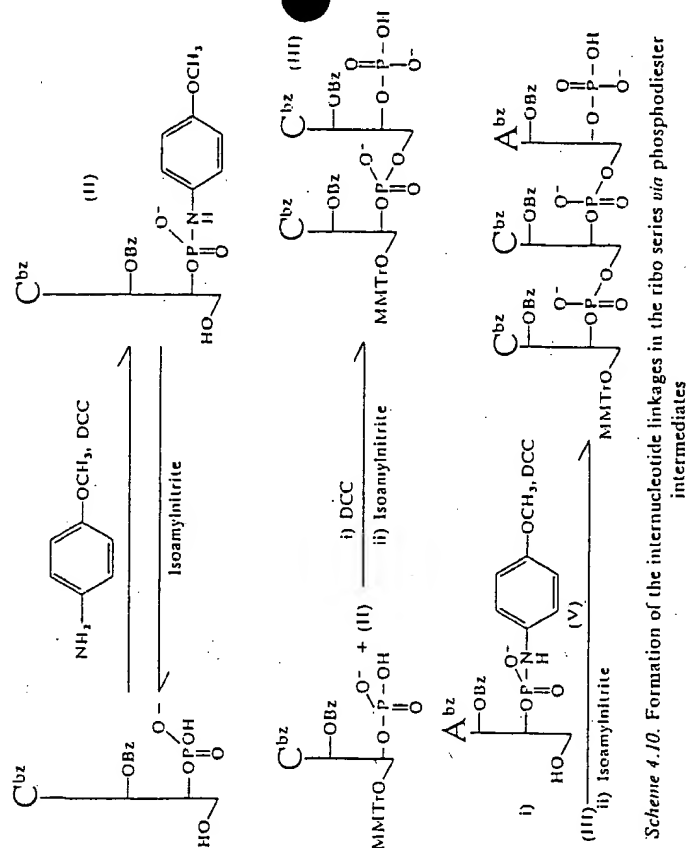


Scheme 4.9. Formation of the internucleotide linkage in the ribo series via phosphodiester intermediates

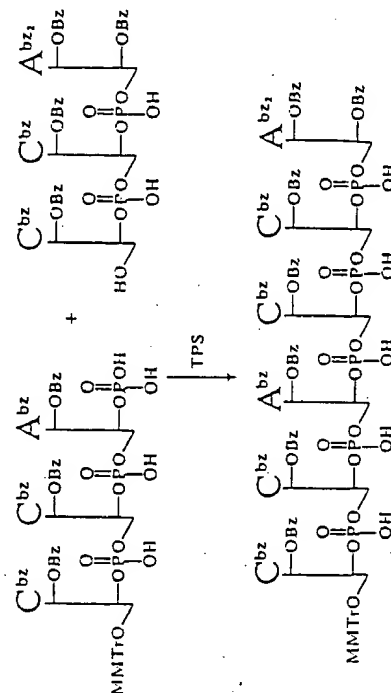
Thus, in contrast to the diester approach in the deoxy series, where removal of an alkali labile 3'-O-acyl group allows further extension at the 3'-end of the growing chain, growth direction in the ribo series

References, pp. 483-508

occurs towards the 5'-end of the chains at which an acid labile trityl group is removed after each condensation step in order to allow further extension.



Scheme 4.10. Formation of the internucleotide linkages in the ribo series via phosphodiester intermediates



Scheme 4.11

While this principle has been used for trinucleoside diphosphate (249, 319) and trinucleotide (313, 319) synthesis, more recently a new approach leading to the opposite growth direction could also be developed. According to this principle a suitably protected 3'-phosphate is condensed with the free 5'-hydroxyl of a 3'-phosphoranosidate derivative (309, 310, 319, Scheme 4.10). The resulting protected dinucleoside is then selectively unblocked at the 3'-terminal phosphate residue by treatment with isoamylnitrite whereupon a subsequent condensation step can be carried out with the free 3'-terminal phosphate residue and the free 5'-hydroxyl of a second nucleosidic component.

This new principle verified by the synthesis of the trinucleotide CpCpAp in a protected form such that further condensation at the 3'-terminal phosphate is possible (Scheme 4.10), appears of considerable value for the preparation of oligonucleotide blocks. In a similar approach, but using a 2',3'-cyclophosphate as protecting group for the 3'-terminus, the trinucleoside diphosphates GpUpA and CpGpUp have been synthesized (311, 314, 319).

While the two approaches mentioned, are based on the stepwise addition of one mononucleotide unit at a time, successful block condensation by the diester method has also been reported now in the ribo series (313, 318). Thus, when the protected trinucleotide $r[(MMTrO)bzC(Obz)-bzC(Obz)-bzA(Obz)p]$ was condensed with the protected trinucleoside diphosphate $r[bzC(Obz)-bzC(Obz)-bzA(Obz)]$ in the presence of TPS, the hexanucleotide $r(C-C-A-C-C-A)$ in the protected form could be isolated in reasonable yield (Scheme 4.11). This hexanucleotide could be used (after removal of the 5'-O-protecting group) for further block addition to the nonanucleotide $r(C-G-U-C-C-A-C-C-A)$ in the protected form (318).

These ribooligonucleotides constitute 3'-terminal sequences derived from certain tRNA species such as yeast alanine tRNA, *E. coli* tyrosine tRNA and others. The nonanucleotide represents the longest ribooligonucleotide of specific sequence synthesized chemically by the diester approach (319).

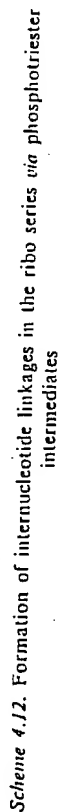
Polycondensation of the protected trinucleotide $bzC(Obz)-bzC(Obz)-bzA(Obz)p$ in the presence of TPS has been tried with limited success (313). While the expected hexamer could be isolated in rather low yield, no nonanucleotide with the repeating sequence could be detected among the reaction products. A systematic study of the polycondensation conditions on mononucleotides and dinucleotide blocks seems therefore desirable before this method can be evaluated more thoroughly.

References, pp. 483—508

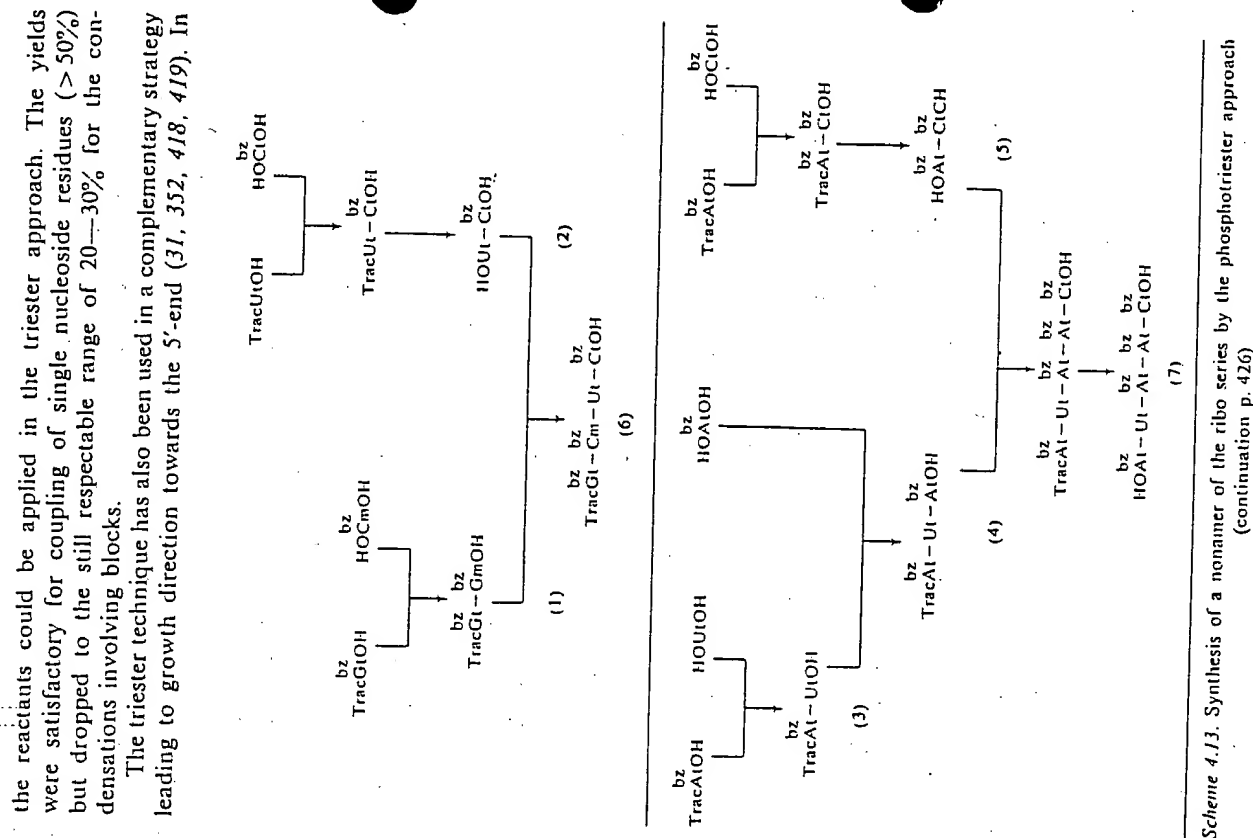
4.1.2.2. Synthesis via Triester Intermediates

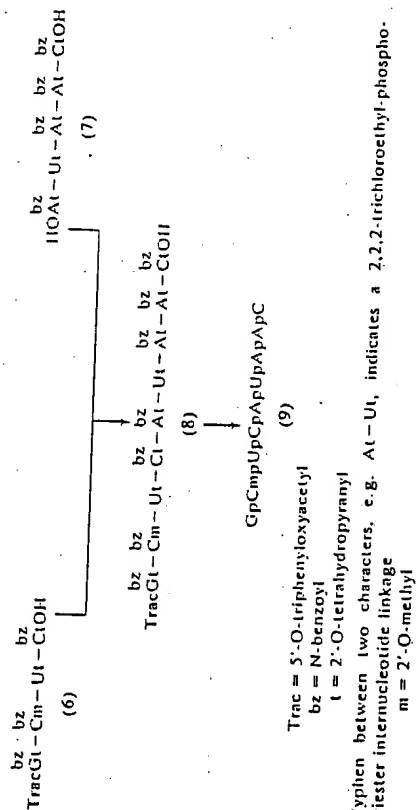
As in the deoxy series the triester approach was expected 1. to suppress side reactions which occur in the diester synthesis due to the reactivity of the diester internucleotide linkages and 2. to allow rapid isolation and purification of the intermediates by extraction procedures, preparative thin layer and/or short column chromatography. After encouraging results were obtained in the deoxy series a number of attempts were therefore undertaken to extend the triester approach also to the ribo series. Benzyl (419), phenyl (31, 352), *o*-chlorophenyl (31), trichloroethyl (296, 298, 299, 300, 418, 419, 462) and β -cyanoethyl (113, 417, 418, 419) groups have been used for the protection of internucleotide phosphate residues and for the conversion of the 3'-terminal phosphomonoester residues, to the corresponding phosphodiester residues. The (Scheme 4.12), after phosphorylation of 5'-O-monomethoxytrityl-2'-C tetrahydropyranlyridine (2) with trichlorethylphosphate in the presence of TPS to give the diester (3) further reaction of (3) with 2'-O-tetrahydropyranlyridine (1) in the presence of TPS leads to the dinucleoside monophosphate derivative (4). As observed consistently also in analogous cases with other nucleoside derivatives (31, 113, 296, 298, 299, 300, 462) condensation of (3) with the 3'-hydroxyl-group of (1) to give products containing 3'→3' phosphodiester linkages in the deprotected compounds could not be detected. Apparently the steric hindrance of the neighbouring 2'-O-tetrahydropyranlyl group and/or of the bulky arylsulphonic-phosphoric anhydride intermediate, together with the secondary nature of the 3'-hydroxyl group, do not allow reaction at this functional group. Consequently no special 3'-hydroxyl protection is necessary and subsequent reactions can immediately be carried out as outlined in Scheme 4.12 for the synthesis of uridyl-(3'→5')-uridyl-(3'→5')-uridine (5). Virtually the same approach seems also feasible when methoxytetrahydropyranlyl group and phenyl or *o*-chlorophenyl groups are chosen for protection of both the 2'- and 5'-hydroxyl functions and the phosphodiester linkages (31).

This stepwise approach, in which overall direction of the chain growth towards the 3'-end results, could also be applied to the synthesis of ribooligonucleotides containing all the four common base residues (298, 299, 300, 462). More recently this principle could even be extended to the condensation of preformed blocks (300, 462) as outlined in Scheme 4.13 for the synthesis of the nonamer GpCmpUpCpApUpApApC (300). The latter corresponds to a sequence occurring in the anticodon loop of tRNA^{Phe} from *E. coli*; this sequence represents the longest ribooligonucleotide of specific sequence synthesized chemically by the triester approach. Protected di- or triribonucleotides (1, 2, 3, 4 and 5) were



assembled stepwise from their 5'-termini starting from 5'-O-trityl-oxyacetyl-2'-tetrahydropyranyl nucleosides by the two-step procedure using trichloroethylphosphate and TPS analogs to the reaction pathway outlined in Scheme 4.12 for the synthesis of UpUpU. Block phosphotriester synthesis of the nonaribonucleotide derivative (8) was then accomplished using a similar procedure from protected tetranucleotide (6) and pentanucleotide (7) which had been the coupling products of dinucleotide derivatives (1 and 2) and of trinucleotide (4) and dinucleotide derivative (5) respectively. It is interesting to note that in contrast to the diester approach, in which increasingly large excesses of the incoming nucleotidic components have to be used in order to achieve satisfactory yields, almost equimolar proportions of





Scheme 4.13. Synthesis of a nonamer of the ribo series by the phosphotriester approach (continued from page 425)

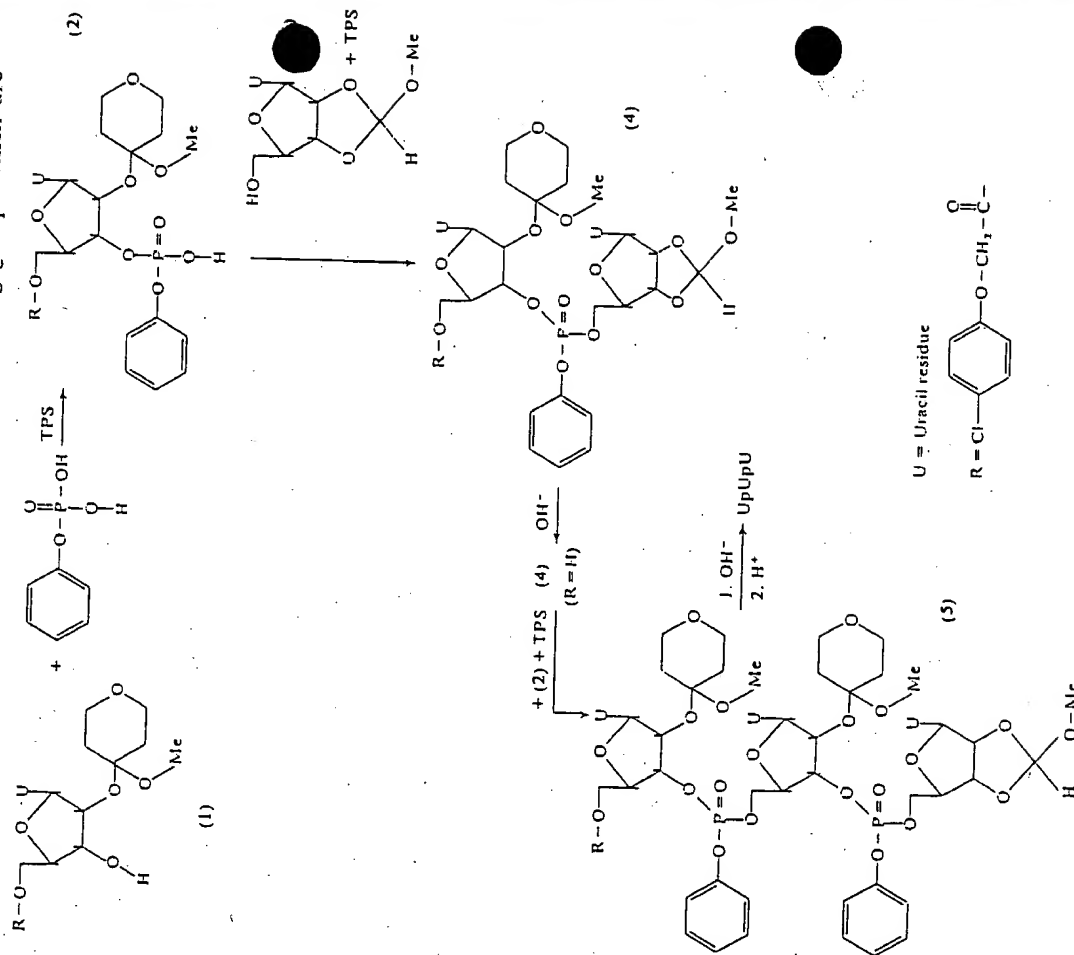
this case, as outlined in Scheme 4.14, the 5'-hydroxyl group of a partially protected nucleoside derivative (3) is reacted with the 3'-diester residue of a protected nucleotide derivative (2) (the latter as usual is prepared *in situ* by reaction of the free 3'-hydroxyl function of a partially protected nucleoside derivative (1) with phenyltrichloroethyl- or β -cyanoethyl-phosphate). The resulting protected dinucleoside monophosphate derivative (4) after selective deprotection of the 5'-hydroxyl function can be further condensed with a second 3'-terminal phosphate containing component (2) to give the protected trinucleoside diphosphate derivative (5).

The use of acid labile groups for the protection of the 3'-terminus and of the 2'-hydroxyl functions necessitates alkali labile protecting groups such as acetyl, formyl, benzoyl or *p*-chlorophenoxyacetyl groups for 5'-O-protection. Selective cleavage of the latter groups is, however, difficult, when the alkali labile β -cyanoethyl group is used for phosphate protection. On the other hand, use of the alkali stable trichloroethyl group leaves the protection of the internucleotide phosphates intact. In contradiction to other reports, however, removal of the trichloroethyl group after the final step seems to be far from quantitative (418). Nevertheless, synthesis up to the tetrauridine triphosphate in reasonable yields could be achieved (418) and the synthesis of ribooligonucleotides containing the other three common bases seems also to be feasible by this approach (419).

One general drawback of the triester approach as compared to the diester technique consists in the longer reaction times required for

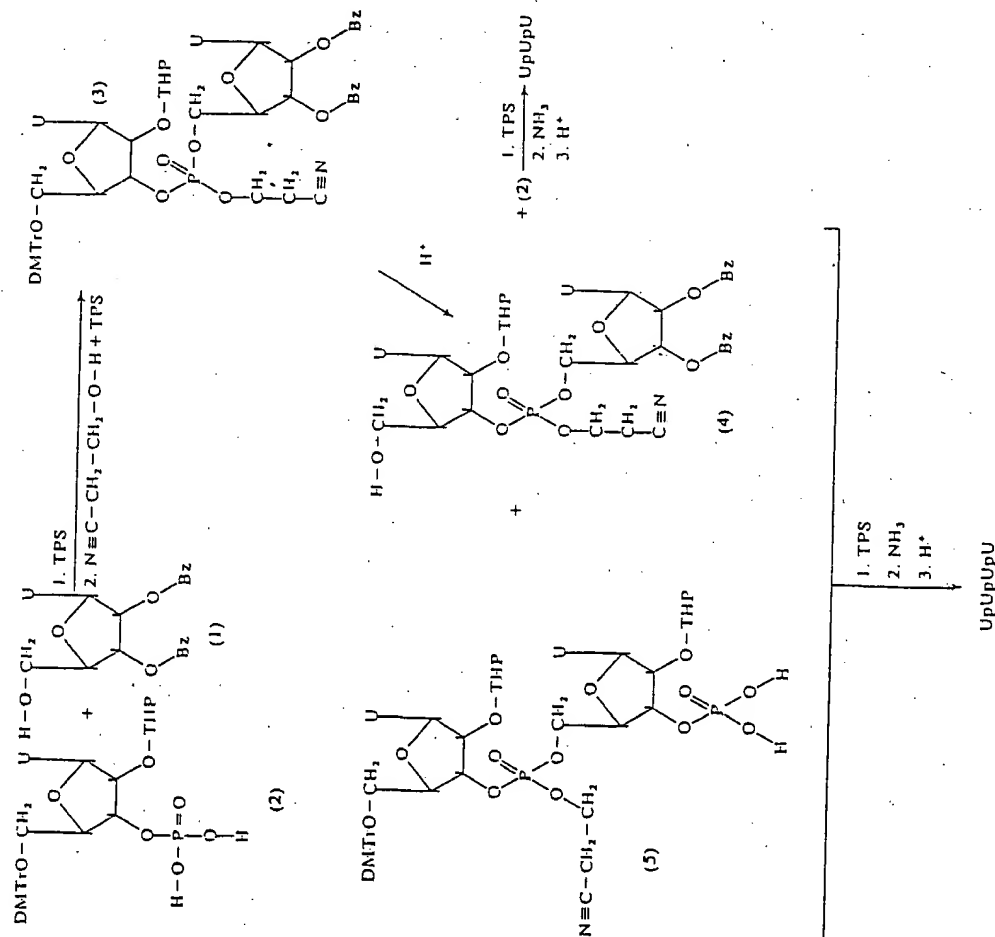
References, pp. 483—508

activation and reaction of the diester intermediates. This relative inertness of diesters (as compared to monoesters) is further increased in the ribooligonucleotide series by the bulky 2'-protecting groups which are



Scheme 4.14. Formation of internucleotide linkages in the ribo series via phosphotriester intermediate

immediately adjacent to the phosphate residues to be activated. An approach has therefore been proposed which would combine the advantageous features of the diester and the triester synthesis (417). Accordingly the internucleotidic bond is first formed from a sterically more favourable and more reactive phosphomonoester component and is then protected *in situ* by the β -cyanoethyl group as outlined in Scheme 4.15.



Scheme 4.15. Formation of internucleotide linkages in the ribo series by the mixed diester-triester approach

References, pp. 483–508

Thus, for the synthesis of oligouridylic acids, 2',3'-di-O-benzoyluridine (1) is first condensed with 2'-O-tetrahydropyran-5'-O-dimethoxytrityluridine 3'-phosphate (2) in the presence of TPS. The resulting mixture is subsequently treated with cyanoethyl in the presence of more TPS, to give the triester (3). After selective removal of the dimethoxytrityl group with mild acid (whereby the tetrahydropyran-5'-hydroxyl group is left intact) the 5'-hydroxyl function of (4) is ready for the second condensation step with (2) or (5).

The yields obtained in the stepwise synthesis of tri- and tetranucleotides by the use of this mixed diester-triester approach seem to compare favourable with the yields obtained by application of the pure diester technique; a more detailed investigation including the synthesis of oligonucleotides containing also the three other common bases seems, however, desirable for the final evaluation of this method. An encouraging yield of 77% could be obtained, when the principle was applied to the condensation of two dinucleotide blocks (Scheme 4.15; 5 + 4 \rightarrow UpUpUpU; 421, 422).

4.1.3. Modified Oligonucleotides

A variety of modified oligonucleotides has been synthesized to permit study of their physicochemical, biochemical and/or biological properties. Comparison of the modified oligomers with the natural compounds is expected to clarify certain biochemical or biophysical aspects that deal with the significance of the various functional groups in polynucleotides. In some cases model compounds have facilitated the use of physicochemical methods for the investigation of polynucleotides.

Modifications have been introduced at the phosphate groups, at the sugar moieties and on the base residues, respectively, in the ribo series as well as in the deoxy series.

Methyl and ethyl phosphotriester derivatives of TpT and of d(ApA) have been synthesized in order to perform pmr, CD and UV spectroscopic studies in organic solution (261). In order to study the properties of dinucleoside monophosphates containing unnatural internucleotide linkages, thymidyl-(3' \rightarrow 3'), and (5' \rightarrow 5')-thymidine have been synthesized (282a, 358). Oligothymidylic acids containing internal pyrophosphate linkages (384) and oligouridylic acids containing an internal 5' \rightarrow 5'-linkage (416) have been synthesized in order to test their primer function for enzymic reactions catalyzed by polymerizing enzymes. As regards altered internucleotide linkages, a number of ribonucleoside monophosphates and higher oligomers containing

2'→5'-internucleotide bonds have been synthesized or isolated as side products (15, 222, 223, 265, 323, 336, 337, 338, 346, 376, 430, 431, 432, 433, 455, 456, 459). A "diuridine monophosphate" containing one arabinoside residue instead of a ribose residue has been synthesized as an oligonucleotide in which the sugar moiety is altered (305). Phosphoramidate analogs of oligothymidylic acids have been prepared as a class with a modified phosphate-sugar linkage, which is susceptible to cleavage by mild acid (237). In addition to this, synthesis of L-adenylyl-(3'→5')-L-adenosine and of L-adenylyl-(2'→5')-L-adenosine (438) has opened a route to oligonucleotides containing modified sugar moieties as well as unnatural internucleotide linkages. Chemical polymerization of ds⁴TMP has led to a mixture of oligo-4-thiothymidylic acid (13) as an oligomer in which the base residues are modified. In addition, preparation of diribonucleoside monophosphates containing 4-thiouridine has been reported (266, 370). Finally, synthetic dinucleoside monophosphates containing adenine 8-thiocyclonucleosides constitute a type of oligomers in which the modification involves both, base residues as well as the sugar moieties (453).

A considerable number of modified oligonucleotides has been derived by chemical modification reactions of natural polymers; as these techniques generally do not involve formation of new internucleotide linkages a survey of these contributions appears to lie beyond the scope of the present review. The numerous modified polymers synthesized by enzymic reactions will be surveyed in one of the following chapters.

4.2. Polymer-Support Synthesis of Oligonucleotides

4.2.1. General Reaction Principle

Synthesis of polypeptides and polynucleotides of defined sequence consists basically of a repetition of similar reaction steps with dissimilar monomeric reactants. For multistep reactions of this type R. B. MERRIFIELD (257) and R. L. LETSINGER (226) have developed the technique of polymer support synthesis. The essential reaction steps are:

1. Attachment of the initial monomer of the projected sequence to a polymeric carrier,
2. Blocking of unreacted functional groups of the carrier and deblocking of the grafted monomer.
3. Chain elongation by a blocked monomer unit.
4. Deblocking of the newly attached monomer.
5. Repetition of steps 3 and 4, until the desired sequence is finished.
6. Cleavage of the product from the support.

References, pp. 483—508

The polymer support method has a number of advantages over the conventional technique of condensation in solution: a) Throughout the chain elongation the growing polypeptide or polynucleotide chain is bound to a polymer. The reaction is, therefore, heterogeneous; problems due to solubility differences among the monomers and between them and the growing chain are eliminated. b) The separation and purification procedures during intermediate reaction steps are reduced to simple washing, filtration or precipitation procedures. The time needed for one chain elongation step is greatly reduced. c) The simplicity of separations allows the use of reactants in big excess or repeated treatment, in order to obtain optimum yields of the desired product. d) The repetition of similar or identical reactions allows the automation of the steps of support synthesis.

However, the method also has several drawbacks: a) Unless the yields of all reaction steps are quantitative, a series of homologous truncated sequences are attached to the support along with the desired sequence. The separation of these unwanted sequences after removal from the support can be very difficult, if not impossible in the case of longer chains (468). b) The reactions can be influenced, even controlled by the diffusion of the reactants through the support matrix. Steric hindrance can further inactivate the support bound material and decrease the reaction rates (215, 231). c) The reactions can be influenced by solvation properties of the carrier (395, 396a).

The method of support synthesis has developed very rapidly in the peptide field. Several biologically active polypeptides have been synthesized in this way, such as bradykinin, insulin A and B chains and ribonuclease. However, a great deal of criticism has also arisen due to the fact that long-chain polypeptides synthesized in this way, in spite of their biological activity are not chemically pure compounds.

In oligonucleotide synthesis the success of the support method has been limited due to the complexity of the reactions involved in internucleotide bond formation and the moderate yields which are generally obtained. Difficulties encountered in separating the cleaved product due to these unsatisfactory yields, has precluded the synthesis of longer polynucleotide chains and no attempts at automation of the process have been made.

4.2.2. Requirements for Supports and Reactants

A) Polymeric Carriers:

The supports are in most cases synthetic macromolecular substances, e.g. polystyrene, but also biopolymers, e.g. polypeptides, or inorganic polymers, e.g. silicagel. As a first approximation these carriers should

be inert substances possessing suitable anchoring groups for an oligonucleotide chain, which reacts freely as if in solution. This picture, however, is an inadequate simplification. Carrier syntheses are complex heterogeneous reactions which have several special features. One of the reactants is immobilized inside a solvated gel or polymer coil, an "immobile" or, according to MERRIFIELD, a "solid" phase. The different molecules of this reactant are in a different environment as to steric hindrance by the surrounding polymer chains and as to accessibility to other reactants penetrating inside from the mobile phase. Once the reaction has taken place unused molecules of mobile reactant as well as mobile reaction products will have to find their way back into the mobile phase. Thus chemical reaction and diffusion processes of various species occur side by side, the overall rate being reaction-controlled for some and diffusion-controlled for other molecules of the immobilized reactant. This is evident from the fact that the rate curve during the initial phase is nearly equal to the one found for the carrier-free reaction, but subsequently, deviates more and more. The overall conversion may also be lower than in a parallel reaction in solution, i.e. some of the reactant molecules may be completely inaccessible.

A basic problem in support synthesis is therefore the optimization of the properties of the support with regard to steric hindrance and accessibility of the immobilized reactant. Attempts to solve this in several ways have been made. The initial approach of R. B. MERRIFIELD (257) was to use swellable, homogeneously crosslinked gels. The degree of swelling (mostly about 2%) is chosen so as to ensure good swelling as well as mechanical stability. Since the degree of swelling can only be increased within certain limits, an alternative was to try to attach the immobilized reactant to the surface of pores and cavities of macroreticular, i.e. heterogeneously crosslinked gels (68). These gels are rigid, non-swellable and relatively insensitive to differences in the solvating power of different media. However, here, too, a compromise has to be found between pore size and mechanical stability. Besides this, the materials used contain a distribution of pore sizes around an average value. Therefore, part of the reactant will be in small pores, i.e. in less readily accessible places. This risk is reduced by using microgels, which are very small rigid gel particles of diameter $0.1-1\ \mu$ (274). In these ratio of outer to inner surface is greatly increased; however, the small particle size precludes the application of simple filtration processes.

In contrast to this development where the immobilized reactant was attached to the surface of a rigid lattice, a concurrent line of development aimed at the use of systems promising maximum chain flexibility. The simplest solution in this direction was the use of linear, non-cross-

References, pp. 483-503

linked polymers as carriers. The first support system of this kind, a chloromethylated polystyrene, was described for peptide synthesis by M. M. SHERYAKIN and coworkers (404). Due to the internal Brownian movement of the polymer chain, the reactant molecules, which are grafted to it will continually change from positions more to the inside of the coil to others more to the outside and back. Diffusion problems are, therefore, at minimum in this case. Unfortunately, the non-cross-linked polymers do not allow filtration techniques. Removal of mobile reactants and products has to be carried out by precipitation or dialysis. This causes problems as the result of solubility changes, secondary crosslinking and adsorption, which will be discussed later in this section.

A third approach, which combines utmost chain flexibility with an insoluble system, was first studied by R. L. LETSINGER and coworkers (226) concurrently for the synthesis of oligopeptides and oligonucleotides. This approach utilizes the so-called popcorn polymers which form on proliferous thermal bulk polymerization of suitable monomers, e.g. styrene. These, too, are heterogeneously crosslinked polymers containing regions of high resp. low coil density. Popcorn polymerization occurs at very low content of divalent monomer ($\sim 1\%$), and crosslinking is effected more by physical than by chemical means. Since the chains are only slightly linked to each other, the chain flexibility in appropriate solvents is nearly as high as in non-crosslinked polymers (34), although the popcorn polymer is insoluble, easily filtrated and mechanically stable. Although more information still has to be collected, it seems that this type of polymer is especially well suited for support purposes in oligonucleotide chemistry.

Another consideration concerns the chain solvation properties of the polymer support. The different steps of oligonucleotide synthesis are carried out almost exclusively in two kinds of media, i.e. pyridine and aqueous or partly aqueous solutions. Especially in the latter case good solvation cannot be expected for supports based on polystyrene. Recent developments in oligonucleotide support synthesis, therefore, aim at the construction of more hydrophilic carriers (33, 44, 392, 396a). Unfortunately, however, all the systems tested so far show a strong tendency to adsorb oligonucleotide chains in aqueous media, thus restricting the utilization of this approach. Cellulose carriers, which show excellent solvation and little adsorption in aqueous media cannot be employed for stepwise oligonucleotide synthesis due to their residual hydroxyl groups. However, they have been very profitably used for enzymatic syntheses (Section 4.2.4) and affinity chromatography (Section 4.2.5).

B) Reactants:

We have distinguished between two types of reactants — the immobilized one, i.e. the growing oligonucleotide chain, and the mobile ones, namely e.g. nucleotide monomers and blocking or deblocking reagents. For the immobilized reactant the polymer can be looked at as a macromolecular blocking group. Since cleavage from the support is the last of all steps leading to the synthesis of a sequence this linkage must be the most stable, and this has to be taken into account on designing a blocking scheme for the intermediates as shown in Section 1.6. From the earlier discussion of the steric influence of the support lattice it is clear that support reactions will be significantly slowed down, when one of the reaction partners is sterically hindered or bulky. This can be seen, for example, on comparing the yields of phosphorylation vs. internucleotide bond formation under comparable conditions. In order to obtain fair yields in a reasonable time it is often necessary to use elevated temperatures and an enormous excess of the mobile reagent (238). This may be a disadvantage, if the reagent is expensive or not readily accessible. On the other hand, the possibility of shifting a reaction equilibrium towards the desired product by using a high excess of mobile reagent or by several repetitions of a reaction cycle may be a reason for using the support approach. It also possesses certain advantages in handling small quantities of reactants.

4.2.3. Chemical Synthesis of Oligonucleotides on Supports

After discussing some general aspects we shall now briefly review the different approaches described for the synthesis of oligonucleotides. Table 4.2 gives a survey of the different methods that have been developed. The methods are classified roughly according to the types of supports used (column 2). The functional groups and the way in which the initial member of the oligonucleotide chain is linked are shown in columns 3 and 4. In the next column the types of intermediates used for oligonucleotide synthesis are described. If necessary, the reaction conditions are indicated. Column 6 gives the conditions for the cleavage of the oligonucleotides from the support. Information on sequences and yields that were obtained is given in column 7. Since it is impossible to list all products, examples are given of a dinucleotide or dinucleoside phosphate, usually the one synthesized in the best yield, and of the longest chain which was obtained by this method. The corresponding references are listed in the last column.

References, pp. 481—508

Table 4.2. Approaches to the Synthesis of Oligonucleotides on Polymer Supports

No. Support	Functional groups	Type of linkage	Conditions and Cleavage intermediates	Sequences, Yields*	References
-------------	-------------------	-----------------	---------------------------------------	--------------------	------------

1	polystyrene, non-crosslinked		pdt-OAc, pdc-OAc, pdg-OAc, acid in triethyl ether	dT(pdT) _n , n = 1-2 sequences dinucleotide dT(pdT) ₂ : 96% dT(pdT) ₃ : 83%	(140, 141)
---	------------------------------	--	---	---	------------

2	polystyrene, non-cross-linked		nucleoside-5'-pdt-OAc	50% trifluoroacetic acid/ dioxane 1:100 dT(pdT) ₂ : 11%	(67)
---	-------------------------------	--	-----------------------	--	------

* Yields given are: best yield of one internucleotide bond formation; overall yield of longest sequence.

Table 4.2 (continued)

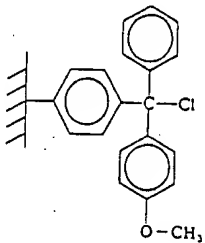
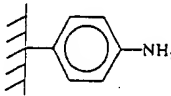
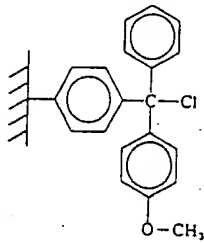
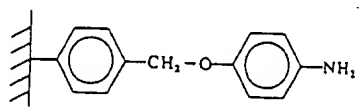
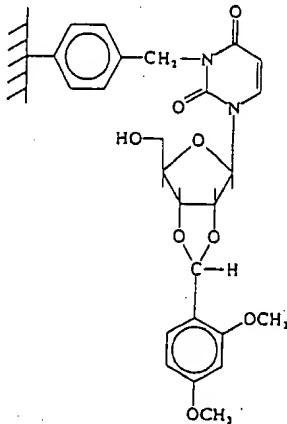
No. Support	Functional groups	Type of linkage	Conditions and intermediates for oligonucleotide synthesis	Cleavage conditions	Sequences, yields*	References
3 polystyrene, non-cross-linked		nucleoside-5'-ether	pdT-OAc 1. POCl ₃ /acetonitrile 2. dT-OAc	trifluoroacetic acid in chloroform	dTpdT 67%	(171, 343)
3a polystyrene, non-cross-linked		nucleotide-5'-phosphoramidate	pdT-OAc	80% acetic acid		(67, 393)
4 polystyrene, gel, 1% X		nucleoside-5'-ether	pdTOAc pdA ^{bz} , pdA ^{ac} OAc pdC ^{ac} , pdC ^{ac} OAc pdG ^{ac} , pdG ^{ac} OAc	acetic acid/water/benzene 32/8/10	dT(pdT) _n n = 1-4 di- and trinucleotide sequences dTpdT: 75% dT(pdT) ₄ : 6%	(254, 255, 256)
5 polystyrene, gel		nucleoside-5'-phosphoramidate	pdTOAc	3x80% acetic acid, 24 h., rt.	(pdT) _n n = 2-3 (pdT) ₂ : 40% (pdT) ₃ : 5%	(22, 23, 24)
5a polystyrene gel, 2% X		nucleotide-5'-uridinyloxy	pdT-OAc	1. acid 2. periodate 3. alkali		(390)

Table 4.2 (continued)

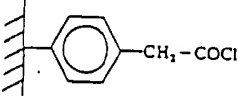
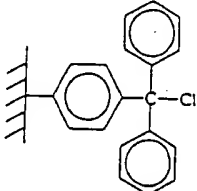
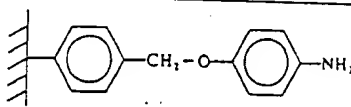
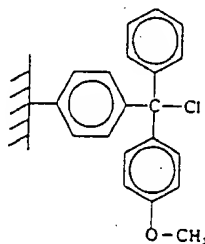
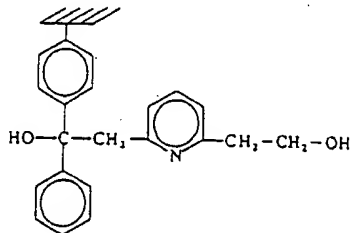
No. Support	Functional groups	Type of linkage	Conditions and intermediates for oligonucleotide synthesis	Cleavage conditions	Sequences, yields*	References
6 polystyrene, gel, 2% X		nucleoside-5'-ester	pdT-OAc pdT-OH	dioxane/ conc. ammonia 1:1	dT(pdT) _n poly- condensate dTpdT 85%	(220)
7 polystyrene, gel 2% X		nucleoside-5'-ether	pdG ⁺ OAc	2% trifluoro- acetic acid in benzene	dT(pdG) _n n = 1-3 dTpdG: 27% dT(pdG) ₃ : ~2%	(476)
8 polystyrene, gel 2% X		nucleoside-5'-phosphoramidate	MMTrU ^{bz} -p MMTrC ^{bz} -p- OBz	isoamyl nitrite in pyridine/ acetic acid 1:1	rApUpGp dinucleotides rApUpGp: 10%	(317)
9 polystyrene, macroreticular		nucleoside-5'-ether	pdT-OAc, pdTpdtOAc, pdC ⁺ -OAc, pdA ⁺ -OAc	80% acetic acid	dT(pdT) _n n = 1-7 di- and tri- nucleotide sequences dT(pdT): 50% dT(pdT) ₇ : 4% dT(TTACCTA): 13%	(68, 215, 217, 218)
10 polystyrene, macroreticular 6% X		nucleoside-5'-phosphomono-ester	pdT-OAc	2N sodium methyrate in methanol/ pyridine 1:1	(pdT) _n n = 2-6 (96) (pdT) ₂ : 35% (pdT) ₆ ~2%	(96)

Table 4.2 (continued)

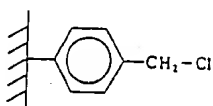
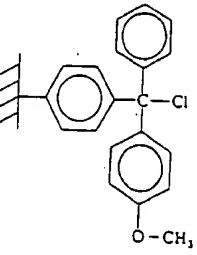
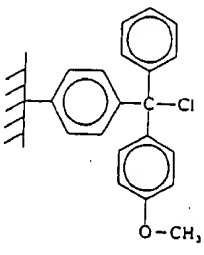
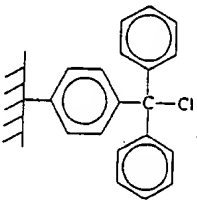
No. Support	Functional groups	Type of linkage	Conditions and intermediates for oligonucleotide synthesis	Cleavage conditions	Sequences, yields*	References
11 polystyrene, macroreticular 5% X		nucleoside-5'-S-benzyl-phosphorothioate	pdTOAc, pdC ^{3'} -OAc, pdA ^{3'} -OAc	I ₂ in pyridine/water 3:1	(pdT) _n , n = 2-5 (423) di- and tri-nucleotide sequences (pdT) ₂ : 39% (pdT) ₃ : <1%	
12 polystyrene, macroreticular ca. 3% X		nucleoside-5'-ether	pdTOAc	80% acetic acid	dT(pdT) _n , n = 1-2 modified dinucleotide dTpdT: 73% dT(pdT) ₂ : 40%	(107)
13 polystyrene, microgel 20% X		nucleoside-5'-ether	—	80% acetic acid	—	(214)
14 polystyrene, gel 40% X		nucleoside-5'-ether	pdA ^{3'} -OAc	2% trifluoroacetic acid in chloroform	dT(pdA) _n , n = 1-3 dTpdA: 80% dT(pdA) ₃ : 43%	(174, 239, 342)

Table 4.2 (continued)

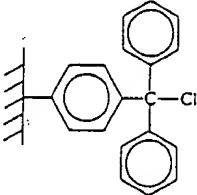
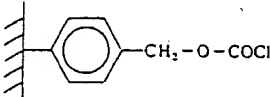
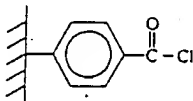
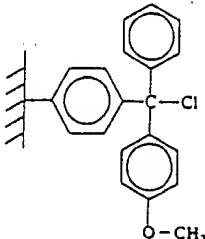
No. Support	Functional groups	Type of linkage	Conditions and intermediates for oligonucleotide synthesis	Cleavage conditions	Sequences, yields*	References
15 polystyrene gel 40% X		nucleoside-5'-ether	1. PCl_3 2. HgCl_2 + nucleotides	2% trifluoroacetic acid in benzene	$\text{dT}(\text{pdT})_n \text{p rU}$ $n = 1-2$ di- and tri-nucleotide sequences dTpdT : 80% $\text{dT}(\text{pdT})_2 \text{prU}$: 23%	(173)
16 polystyrene, popcorn 0.2% X		deoxycytidine-N-carbamate	1. β -cyanoethyl-phosphate, DCC 2. thymidine, MS	0.2 M sodium-hydroxide in dioxane/water 1:1	$\text{dC}(\text{pdT})_n$ $n = 1-3$ dCpdT : 61% $\text{dC}(\text{pdT})_3$: 14%	(226, 227, 228)
17 polystyrene, popcorn 0.1% X		nucleoside-5'-ester	1. β -cyanoethyl-phosphate, DCC 2. blocked nucleoside, MS	0.5 N sodium hydroxide in dioxane/water 1:1	$\text{dT}(\text{pdT})_n$ $n = 1-2$ $\text{dG}(\text{pdG})_n$ $n = 1-3$ di- and tri-nucleotide sequences $\text{d}(\text{GGGT})$ dTpdT : 95% $\text{dT}(\text{pdT})_2$: 78% $\text{dG}(\text{pdG})_3$: 7% $\text{d}(\text{GGGT})$: 18%	(229a, 230, 407, 409)
18 polystyrene, popcorn		nucleoside-5'-ether	pdTOAc , pdTpdTOAc	80% acetic acid	$\text{dT}(\text{pdT})_n$ $n = 1-5$ dTpdT : 64% $\text{dT}(\text{pdT})_3$: 3.5%	(213)

Table 4.2 (continued)

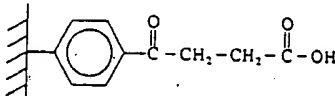
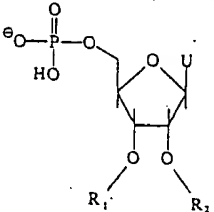
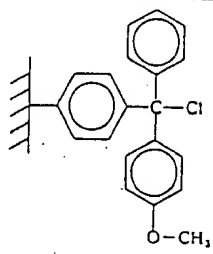
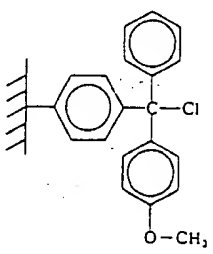
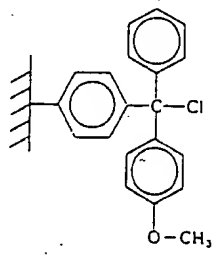
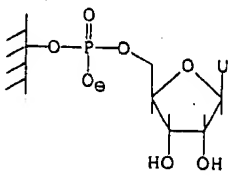
No. Support	Functional groups	Type of linkage	Conditions and intermediates for oligonucleotide synthesis	Cleavage conditions	Sequences, yields*	References
19	polystyrene, isotactic	nucleoside-5'-ester		0.5N ammonia	rU(prU) _n n = 1-2 rUprU: 52%	(470)
						
			R ₁ = methoxy-acetyl R ₂ = benzoyl-propionyl			
20	polystyrene, isotactic	nucleoside-5'-ether	pdTOAc 3'-O-acetyl-5'-iododeoxy-uridylic acid	trifluoroacetic acid in chloroform	dT(pdT) _n n = 1-2 dTpdT: 55% modified dinucleotides	(447)
						
21	polystyrene, isotactic	nucleoside-5'-ether	pdA ^{bz} -OAc	trifluoroacetic acid in chloroform	dT(pdA) _n n = 1-3 dTpdA: 80% dT(pdA) ₂ : 28%	(342)
						
22	polyethylene glycol	nucleoside-5'-ether	—	—	—	(212)
						
		2'(3')-5'-inter-nucleotide linkage	—	—	—	

Table 4.2 (continued)


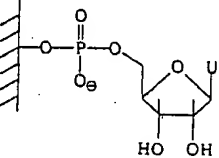

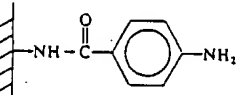
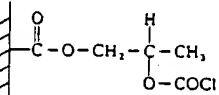
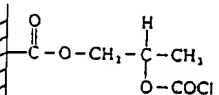

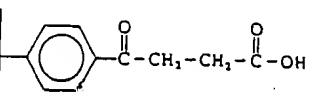
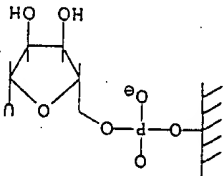
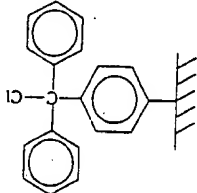
No. Support	Functional groups	Type of linkage	Conditions and intermediates for oligonucleotide synthesis	Cleavage conditions	Sequences, yields*	References
23	α, ω -diamino-polyethylene glycol 	phosphoramidate	pdA ^{tr} -OAc	isoamylnitrite in pyridine/acetic acid 1:1	(pdA) ₃ : 14%	(33)
24	polyvinyl-alcohol non-cross-linked 	2'(3')-5'-inter-nucleotide linkage	pdTOAc	alkali	(pdT) _n , n = 1-5 (pdT) ₂ : 51% (pdT) ₃ : 8%	(33, 378, 382)
25	vinylacetate-N-vinylpyrrolidone copolymer non-cross-linked 	nucleoside-5'-carbonate	1. pdT, poly-condensation 2. pdT-OMMT ^r resp. prU(-OAc) ₂ , TPS	conc. ammonia	dT(pdT) _n , n = 1-3 dT(pdT) _m rU, m = 1-2 dTpdT: 56%	(392, 394, 395, 396a)
26	poly-L-lysine 	nucleoside-5'-phosphoramidate	pdTOAc	isoamylnitrite in pyridine/acetic acid 1:1	(pdT) ₃ : 14%	(44)
27	styrene-acrylic acid copolymer, popcorn 	nucleoside-5'-carbonate	1. β -cyano-ethyl-phosphate, MS 2. blocked nucleoside, TPS	0.5 n sodium hydroxide in dioxane/water 1:1	dinucleotide sequences dTpdT: 92%	(238)
28	Bio Rex 70 (polyacrylic acid, macroporous) 	nucleoside-5'-carbonate	1. β -cyano-ethyl-phosphate, MS 2. blocked nucleoside, TPS	0.5 n sodium hydroxide in dioxane/water 1:1	dinucleotide sequences dTpdT: 65%	(238, 395)
29	Merckogel-10 ⁶ (polyvinyl-acetate, macroporous) 	nucleoside-5'-carbonate	1. β -cyano-ethyl-phosphate, MS 2. dT-O β B, TPS	0.5 n sodium hydroxide in dioxane/water 1:1	dT(pdT) _n , n = 1-4 dTpdT: 59% dT(pdT) ₄ : 5%	(238, 395, 397a)
30	Bio-Beads S-X2 (polystyrene, macroporous) 	nucleoside-5'-ester	1. MeOPOCl ₂ 2. dTOAc	0.5 n sodium hydroxide in dioxane/water 1:1	dT(pdT) _n , n = 1-2 dTpdT: 38% dT(pdT) ₂ : 10%	(306)

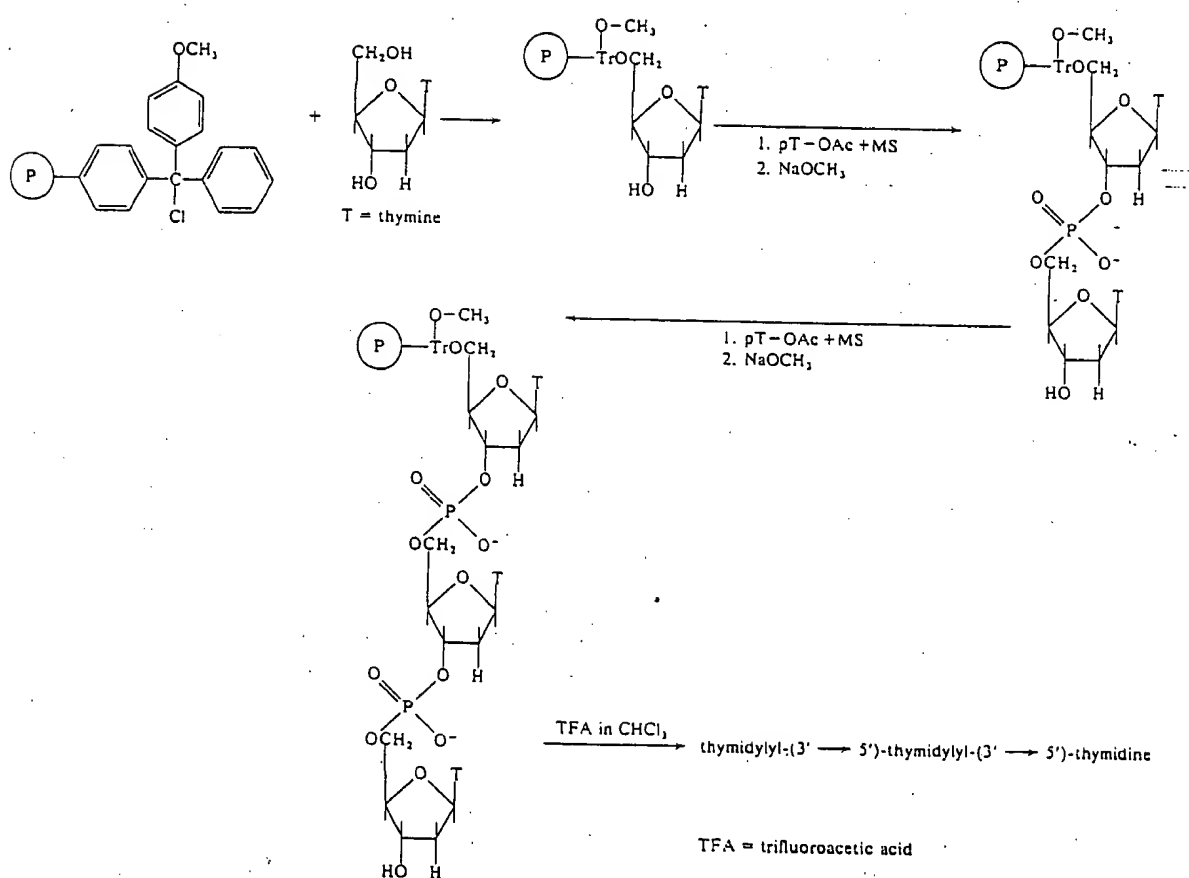
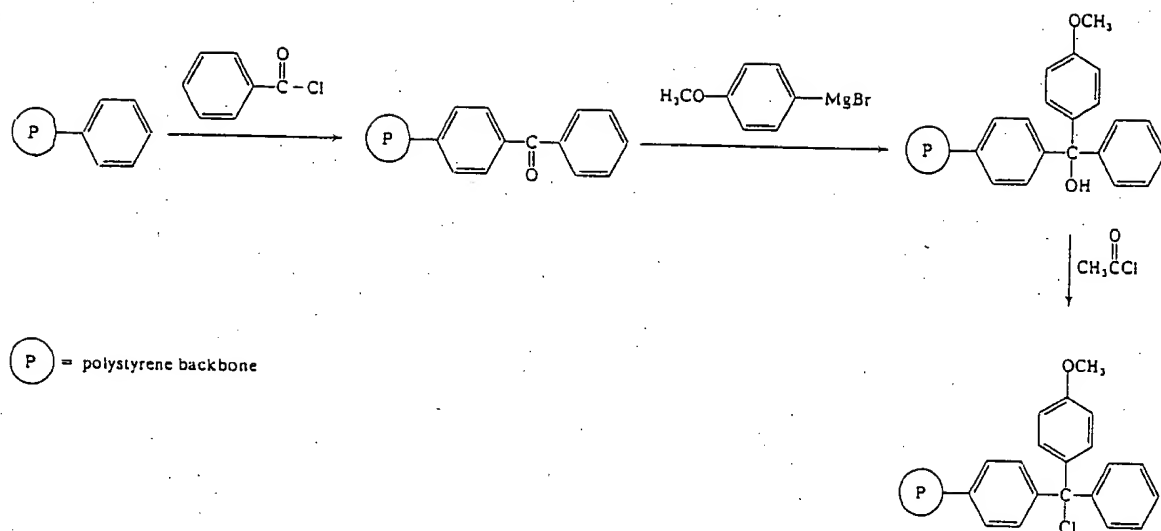
Table 4.2 (continued)

No. Support	Functional groups	Type of linkage	Conditions and Cleavage intermediates	Sequences, yields*	References
31 Sephadex LH 20		2'(3')-5'-internucleotide linkage	0.1 N sodium hydroxide	(211)	
32 Silicagel		5'-ether nucleoside-pdTOAc	80% acetic acid	dTPdT 54% (210)	

The majority of systems developed, 21 out of 32, use polystyrene derivatives as carriers. In most of these cases the supports are derivatized so that they possess trityl chloride groups to which nucleosides can be linked as ethers. This acid-labile linkage allows chain extension according to the reaction scheme developed by H. G. KHORANA and coworkers (see Section 1.6). The conditions for acidic removal of the oligonucleotides have to be carefully chosen, since the support reactions are generally slower (see Section 4.1.2) than in the carrier-free case and the risk of decomposition of the oligonucleotides on prolonged exposure to acid is high. Dimethoxytrityl groups have therefore been used in some cases as anchoring groups (147). They allow a more facile acidic cleavage, however, the linkage is so labile that some nucleotide material may be lost during the washing procedures. The yields of internucleotide bond formation have been highest (>90%) with soluble polystyrene derivatives (no. 1-3) (67, 141, 343), where they reach the maximum yields obtained in carrier-free approaches. Lower yields (50-80%) have been reported for all other types of tritylated polystyrene carriers (no. 4, 6, 7, 9, 12, 14, 20, 21) (68, 107, 218, 220, 256, 342, 447, 476). An example of this type of oligonucleotide support synthesis is given in Scheme 4.16.

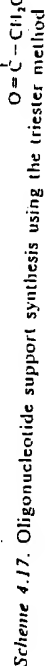
In this context it should be mentioned that a comparison of the different methods is difficult, even if (as in the above mentioned case), the type of linkage is identical and, in most cases the same product, namely a thymidylic acid dimer, was prepared to test the formation of an internucleotide linkage. This is due to the complexity of the parameters of support reactions, and, in fact, only few of these support systems seem to have been so extensively studied as to ensure optimization of most factors.

The difficulties encountered in using an acid-labile support linkage have prompted investigations of other types of anchors, which would allow cleavage of the nucleotidic material from the support in alkaline or neutral media. Alkali-labile linkages are formed on conversion of polystyrene resins to macromolecular acids (no. 19 and 30) (306, 470), acid chlorides (6, 17) (220, 229a) and chloroformates (16) (227, 228). This approach, developed first in the laboratory of R. L. LETSINGER, forbids the use of the alkali-labile acetyl groups for the protection of the growing chain end (i.e. in most cases of the 3'-terminus). Development of new blocking groups, such as the β -benzoylpropionyl group, and the application of the triester method made this approach workable and allowed the preparation of small oligonucleotide fragments in excellent, often near quantitative yields (230). The route for the preparation of a trinucleoside diphosphate is shown in Scheme 4.17.



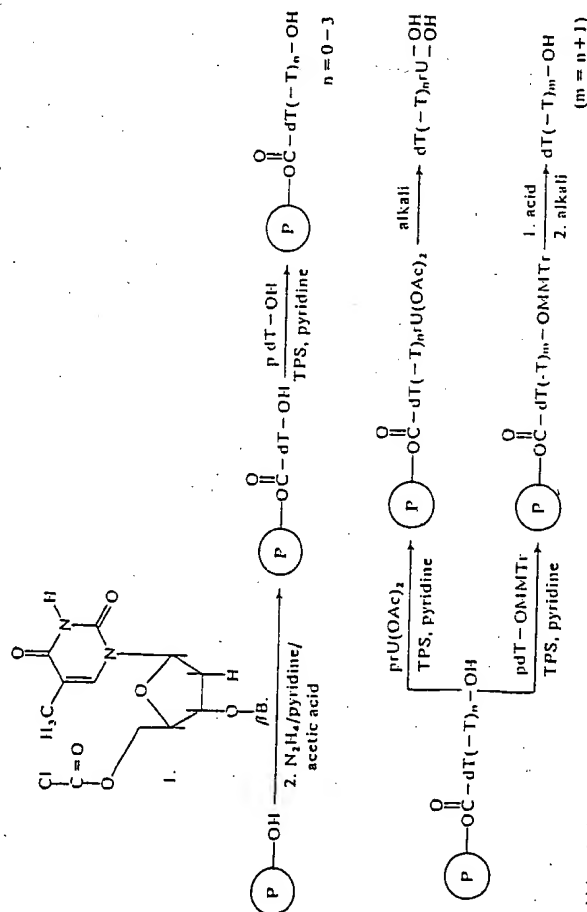
This leads us to another interesting line of development. For some time doubts had arisen as to whether polystyrene resins are really good carriers for oligonucleotide syntheses at all, as polystyrene is most highly solvated in less polar media, whereas the steps of oligonucleotide synthesis are carried out either in water or polar organic media. In order to construct more hydrophilic support resins H. SELIGER and more recently K. K. OGILVIE used either popcorn copolymers of styrene and acrylic acid or commercial hydrophilic macroporous resins as the basis for carriers (238, 306, 395, 397a). Alkali-labile ester or carbonate linkages were used to attach the initial nucleoside and chain elongation was carried out according to Scheme 4.17. Uridylic acid has been used as connection in some instances to procure alkali-labile attachment (no. 22, 24, 31) (211, 212, 378, 382). The yields of internucleotide bond formation were, again, nearly quantitative in the case of the popcorn support (no. 27) (238), whereas the maximum yields obtained with macroporous resins (no. 28, 29, 30) (238, 306, 395) and also with silicagel as porous inorganic support (no. 32) (210) were lower and again in the same range as those obtained previously with macroporous polystyrene. This suggests that the outcome of oligonucleotide reactions is influenced principally by the selection of a certain support structure and only to a lesser extent by better solvation, thus, underlining the importance of steric factors in oligonucleotide support synthesis.

Parallel to the development of more polar insoluble supports non-crosslinked hydrophilic polymers have been modified and tested. These



include polyethylene glycol (no. 22, 23) (33, 212), polyvinylalcohol or saponified vinylacetate-N-vinylpyrrolidone copolymer (no. 24, 25) (33, 382, 392). These approaches differ from the previously described methods using soluble polystyrene (no. 1 and 2) in that the supports are generally soluble in organic media and water, and dialysis methods are used for the separation of mobile reactants (for this reason, the approach, has been named "liquid-phase synthesis" (33)). An example (392, 396a) is illustrated in Scheme 4.18. The yields of oligonucleotides obtained with these systems are still significantly lower than those reported for system no. 1 and also some adsorption difficulties still have to be overcome. Nevertheless, this new technique, which has been successfully applied also in the polypeptide field, should merit further investigation.

Although interest in support synthesis has been focused on the preparation of deoxyoligonucleotides, work in the ribo series has



Abbreviations:

P = vinylalcohol-N-vinylpyrrolidone copolymer

βB = β-benzoylpropionyl

Ac = acetyl

MMTri = p-methoxytrityl

Scheme 4.18. Oligonucleotide synthesis on a soluble support

References, pp. 483-503

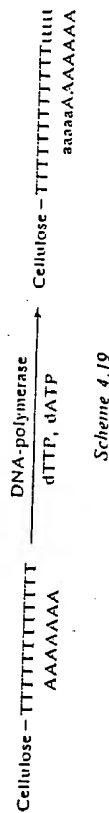
occasionally been described. Thus K. F. Yip and K. C. Tsou (470) have prepared a trimer of uridylic acid, and the sequence rAUG was made by E. Ohtsuka *et al.* (317) using a phosphoranilidate attachment to polystyrene. The carrier method can also serve for the preparation of a modified oligonucleotide, as was demonstrated by the synthesis of two dinucleotides containing 5-fluorouridine (447) and α-pyridone nucleoside (107).

Looking at the results of oligonucleotide support synthesis tabulated in column 7 of Table 4.2, one cannot avoid the statement that, for all the time and effort spent on the development of this technique, the outcome has been relatively meager. Good, even excellent results have been obtained in the synthesis of short oligonucleotide fragments, but here in the meantime the support method has to compete with other effective techniques, such as those using extraction methods or affinity chromatography (see Sections 1.4, 3.1.2, 3.2). For the synthesis of long oligonucleotide chains, because of the low yields of chain extension, it is not feasible to go beyond the range of good chromatographic separability, if one wants to end up with pure compounds. Few support syntheses of longer oligonucleotide chains containing more than one base have been described; among these the blockwise preparation in good yield of a heptanucleotide sequence by H. Köster, A. Pollak and F. Cramer (218) may show that the preparation of fragments for biologically active polynucleotides can, in fact, be done by support techniques. However, unless this technique is significantly improved, the advantage of time saved by avoiding the column chromatography of intermediates is partly compensated by prolonged reaction times, lower yields and relatively difficult separation of the mixture of products released from the carrier.

4.2.4. Enzymatic Synthesis of Oligo- and Polynucleotides on Supports

The attachment of oligo- and polynucleotides to carriers allows the use as support-bound primers and templates in enzymatic reactions. Generally cellulose is used as a support in these cases, since it is highly polar, insoluble and does not interact strongly with polynucleotides. Residual hydroxyl groups do not disturb as long as there is no chemical internucleotide bond formation. The binding of primers and templates to matrices greatly facilitates the removal of the enzymes and excess substrates from the reaction mixture. In the case of cellulose matrices the bound oligonucleotides react as if free in solution. DNA and RNA polymerase, terminal nucleotidyl transferase and polynucleotide ligase were used for chain extension and joining of polynucleotides. Poly-

deoxyadenylate, base paired to poly-dT-cellulose, could be elongated to give support-bound poly dA-dT (170). This is schematically represented in equation 4.19.



Similarly, a natural DNA was elongated by a homopolymer piece by terminal deoxynucleotidyl transferase and base-paired to a complementary oligomer bound to cellulose. DNA polymerase then copied the natural DNA to give a support-bound template for further replications. Poly dC could be joined to poly dC, oligo dT-cellulose in the presence of poly dI by polynucleotide ligase (60). With equally good results ficoll (367b) or polyvinyl alcohol (361a) were employed as carriers in the elongation of support-bound oligonucleotides by terminal deoxynucleotidyl transferase; these chains could subsequently be used as templates for polynucleotide synthesis with DNA polymerase I. Guanosine-2', 3'-cyclophosphate, inserted into a polyacrylamide resin, could be condensed with uridine in a reaction catalyzed by guanyl-RNase (196). Compared to the alternative approach of enzyme fixation to carriers the use of support-bound primers and templates is still in the beginning; it may, however, become very valuable as soon as the techniques for enzymatic synthesis of specific sequences (see Section 5) are further developed.

4.2.5. Miscellaneous Uses of Supports in Nucleotide Chemistry

In the last part of this section we will discuss some uses of polymer supports pertaining to oligonucleotide synthesis which do not, however, involve internucleotide bond formation with a support-bound nucleic acid constituent.

Phosphorylations of nucleosides bound to supports have already been described as part of oligonucleotide syntheses according to the triester method (see Scheme 4.17). Similarly, conversion of nucleosides to nucleotides in good yield have been described by M. M. KABACHNIK *et al.* using tritylchloride carriers and either halides of phosphoric or pyrophosphoric acid or $\text{PCl}_3/\text{HgCl}_2$ as phosphorylating agents (171, 172).

G. M. BLACKBURN and coworkers (22, 23, 24) have used supports for clarifying the mechanism of phosphorylation by dicyclohexylcarbodiimide and sulfonylchlorides. In both cases polymeric phospho-

monoesters were not converted to phosphodiester with aliphatic alcohols or nucleosides. It was concluded that trimeric or polymeric phosphate esters must be necessary intermediates in phosphorylations or internucleotide bond formation by the above mentioned reagents. Such intermediates would not be formed readily, if the phosphate component were fixed to a polymer matrix. Other findings, e.g. the near quantitative internucleotide bond formation with support bound nucleotides in the triester synthesis (Scheme 4.17) (230, 238) are not along this line, although these systems differ from the ones used in the mechanistic investigation in that phosphodiester were the starting compounds. A more thorough discussion of phosphorylation mechanisms has been given in Section 2.

In all cases discussed so far, a support-bound nucleoside or nucleotide served as a partner in internucleotide bond formation. Alternatively, the condensing agent can be bound to a polymer. Nucleoside and nucleotide component are then incubated with the polymeric condensing reagent and, after a suitable reaction time, the oligonucleotide is filtered off. This principle has been exploited by M. RUBINSTEIN and A. PATCHORNIK (362) using poly-3,5-diethylstyrene sulfonylchloride as a macromolecular analog of mesitylenesulfonylchloride. 70—90% of dTpdT could be recovered in a test reaction using alternatively the diester or triester version of internucleotide bond formation. Although separation problems are only partly simplified, the advantage of this approach could lie in an approach to quantitative yields of internucleotide bond formation on further amelioration of the conditions and on use of more sophisticated polymeric reagents.

Other uses of support bound nucleic acids and constituents in nucleic acid chemistry, e.g. in affinity-chromatographic separations, will be discussed in a forthcoming review (397).

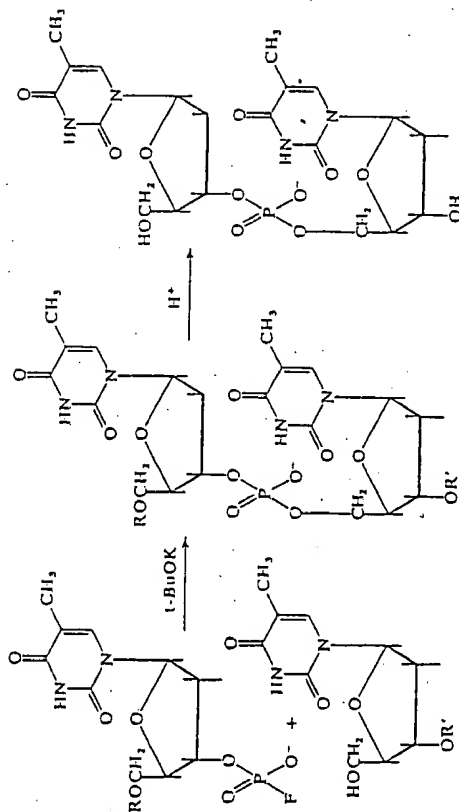
4.3. Miscellaneous Methods in Chemical Oligonucleotide Synthesis

In a search for other condensation methods, experiments have been undertaken which are aimed at the following goals: first, to activate the hydroxyl residue involved in phosphodiester formation; secondly, to use preactivated phosphomonoester derivatives, thirdly to use unprotected or less protected nucleotide derivatives and finally to achieve non-enzymic synthesis on complementary templates.

4.3.1. Chemical Synthesis via Activation of Hydroxyl Functions

Starting from the earlier observation that nucleoside 3',5'-cyclophosphates can be synthesized from the corresponding 3'- or 5'-p-nitro-

phenylesters in the presence of anhydrous strong base a new synthetic route for stepwise synthesis of oligodeoxyribonucleotides has been proposed (440). As outlined in Scheme 4.20, the reaction of suitably



Scheme 4.20. Formation of an internucleotide linkage by activation of hydroxyl groups

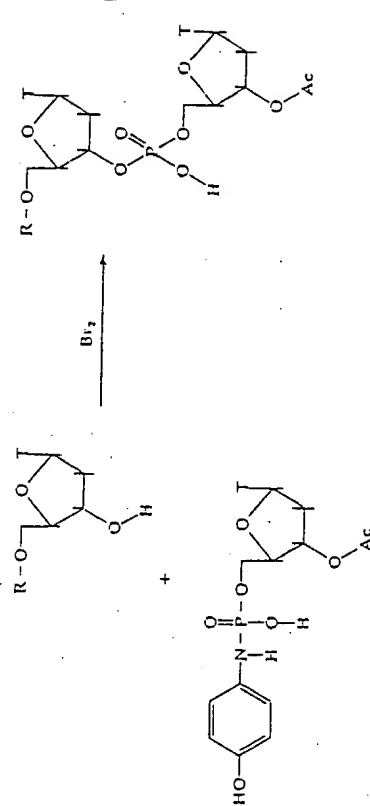
protected nucleosides and nucleotides is carried out with anhydrous potassium tertiary butoxide as the base and fluoride as the leaving group on the phosphate. A stepwise synthesis is possible with the use of protecting groups of different stability in acid. The internucleotide bond was also formed when a secondary 3'-hydroxyl group of the one component attacked a 5'-phosphorofluoridate of the second condensation component. In the oligothymidylic acid series this fast reaction (condensation times range between 15 and 30 minutes) has allowed the stepwise synthesis of the corresponding dinucleoside monophosphate and trinucleoside diphosphate in excellent yields. As demonstrated by the synthesis of d-ApT in 50% yield this reaction principle can also be extended beyond the oligothymidylic acid series although synthesis of oligonucleotides containing C or G residues has not been reported so far. It is noteworthy that in the case of the d-ApT synthesis protection of the base residues was not necessary and that cleavage of the N-glycosidic linkage in anhydrous base apparently does not constitute a serious problem. Further work seems, however, necessary in order to extend this approach to the synthesis of longer chains as in the

References, pp. 483-503

synthesis of TpTpTpT only a 19% yield was observed owing to the insolubility of the potassium salt of TpTpT-MMTri in dimethylformamide (440).

4.3.2. Chemical Synthesis via Preactivated Phosphate Derivatives

Preactivation of phosphomonoester residues can be achieved by amidation with *p*-hydroxyaniline (316). As outlined in Scheme 4.21,



Scheme 4.21. Oligonucleotide synthesis via preactivated phosphate derivatives

in situ activation of 3'-O-acetyl-thymidine 5'-phosphoro-*p*-hydroxyanilide by oxidation with bromine leads to the formation of a protected dinucleoside monophosphate when 5'-trimethylacetyl thymidine offered as hydroxyl component. This principle could be extended to the activation of 3'-monophosphate residues and (in the ribo series) to N-benzoyl-cytosine containing nucleotide derivatives. Since the yields ranged from 24 to 46% for internucleotide bond formation further efforts which would also include attempts at synthesis of longer chains or synthesis of protected adenosine and guanosine derivatives seems desirable.

Polycondensation mediated by preactivated phosphomonoesters has been reported in the oligothymidylic acid series. Thus, activation of thymidine 5'-S-ethyl phosphorothioate (56) with iodine in pyridine in

the absence of any external nucleophile produces extensive self-condensation by attack of the 3'-hydroxyl group on the iodine-activated phosphorothioate. After the polycondensation, conversion of the 5'-terminal S-ethyl-phosphorothioate groups to phosphate groups could be achieved by addition of water and the level of pyrophosphate linkages could be reduced by subsequent acetic anhydride treatment. Oligonucleotides up to the nonanucleotide could be isolated in excellent yields. Although this approach has been reported only for the oligothymidylic acid series so far, it seems reasonable to assume that it can be applied successfully also to other series or to block polymerization.

4.3.3. Chemical Synthesis Using Unprotected Nucleotides

Polycondensation of deoxynucleoside 5'-phosphates is also observed when the disodium salts are refluxed in dry dimethylformamide for 30 minutes (340). This reaction, carried out with unprotected mononucleotides, is catalyzed by protons or proton donors and involves P^1, P^2 -dinucleosidyl-5'-pyrophosphate as a key intermediate. The main products are two series of oligomers with structural formulas of $(pN)_n$ and $(pN)_n p$. However, 5–10% of the oligonucleotides contain at least one 5'–5'-phosphodiester linkage or pyrophosphate linkage as indicated by their resistance to spleen phosphodiesterase. Though, in the oligothymidylic acid series excellent yields up to the nonanucleotide could be observed, the preparative value of this method seems somewhat limited in view of the relatively high proportion of unnatural internucleotide linkages. The study of this polymerization process may, however, provide additional understanding about the prebiotic synthesis of polynucleotides.

In the ribo series polymerization reactions have also been studied under prebiotic conditions. Thus, when excess uridine is heated in the presence of dihydrogen phosphate and urea, di- and oligonucleotides are formed in about 33% yield (323). The majority of the internucleotide bonds formed were 3'–5'-linked; however, large numbers of 2'–5'-bonds and some 5'–5'-bonds were also formed. When adenosine-cyclic 2',3'-phosphate is reacted in the dry state in the presence of aliphatic diamines at moderately elevated temperatures, self-polymerization to give oligonucleotides of chain length up to 6 and higher is observed (455, 456). Here too, the products contain excess of 3'–5'-linkages over 2'–5'-linkages. While the preparative value of these methods seem to be somewhat limited in view of the relatively high frequency of unnatural internucleotide bonds, the study of this polymerization processes may also provide insights into the prebiotic synthesis of polynucleotides.

References, pp. 483–508

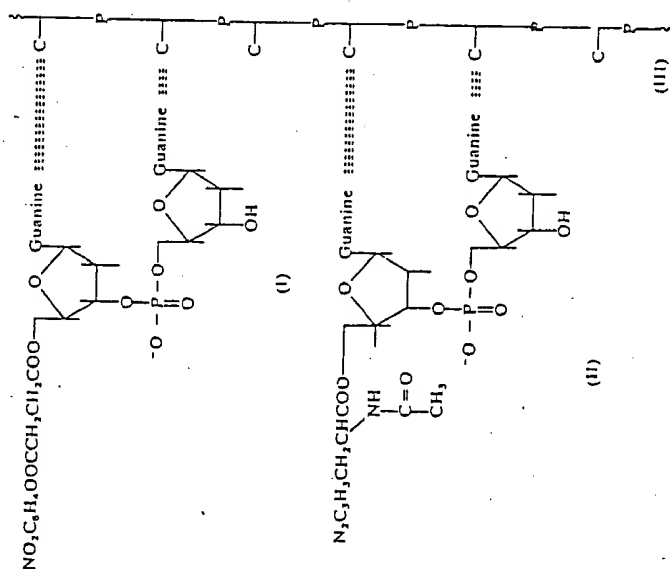
During conventional diester or triester synthesis the amino functions of the base residues are blocked by acylation. The use of derivatives containing free amino functions seems, however, possible in the case of guanine and adenine mononucleotide blocks, at least for the synthesis of dinucleotides in the deoxy series (292, 465) and in the ribo series (265). Whether this simplified approach is also feasible for the synthesis of longer chains remains to be shown. As mentioned previously, N-protecting groups are also unnecessary when condensation reactions are carried out in the presence of strong bases by which the hydroxyl functions are activated (Scheme 4.20) (440). In the triester approach an activated 3'-phosphodiester of one component seems to react exclusively with the free 5'-hydroxyl of the second component, even if the 3'-hydroxyl function of the latter is also unblocked (Schemes 4.12 and 4.14) (31, 296, 300).

4.3.4. Chemical Synthesis on Complementary Templates

This approach tries to mimic enzymic reactions catalyzed by template dependent polymerizing enzymes or by polynucleotide ligases. Accordingly, attempts have been made to combine mononucleotides or oligonucleotides after fixation in mutual vicinity by complex formation on a complementary template. Since the feasibility of this approach was first verified by the synthesis of undecathymidylic acid from two hexathymidylic acid blocks on a poly A template in the presence of a water soluble carbodiimide (294), several other groups have tried to improve this technique for synthetic purposes or to investigate its relevance to prebiotic polynucleotide synthesis. Starting with mononucleotide units in the presence of water soluble carbodiimides as activating agents, poly U and poly C have been tested as templates for the synthesis of oligo A, oligo dA and oligo G respectively. Besides some tri- and tetranucleotides, the main products isolated from the rather complex reaction mixtures consisted of the respective dinucleotides or dinucleoside monophosphates (430, 431, 432). Similar results were obtained with adenosine cyclic 2',3'-phosphate (355) or adenosine-5'-monophosphorimidazole (376, 459) were used as preactivated nucleotide derivatives in the presence of poly U. These reactions seem to be highly specific in respect to base selection by the template, as incorporation of nucleotides not complementary to the template generally is much lower than incorporation of complementary nucleotides. The synthetic value of this monomer approach, however, is severely limited by the observation that – beside other side reactions – the unnatural 2'–5'- and 5'–5'-linkages are frequently formed in preference to the natural 3'–5'-linkages.

When trioxoadenylic acid is reacted with water soluble carbodiimide in the presence of poly U as template at 20 mM MgCl_2 , an overall yield of 35% of the polymeric products d(pApApA)_n ($n=2, 3, 4$) is observed (12). It seems that this rather high yield is due to the presence of Mg^{++} which increases the stability of the $[\text{d(pA)}_3 \cdot \text{poly U}]$ hybrid. When dideoxyadenylic acid or trioxoadenylic acid, activated by 3'-terminal phosphoanidates, are reacted in the presence of poly U, formation of oligo and poly dA is observed (295, 403) in about 10% yield. As evident from degradation with spleen and venom phosphodiesterase, the tetra- and hexaadenylic acids obtained contain natural $5' \rightarrow 3'$ -linkages exclusively (295).

In order to study the arrangement and reactivity of oligonucleotides on complementary templates, a number of G-containing p-nitrophenyl-oligonucleotide succinates (403, 410) have been prepared (Scheme 4.22) and tested for hydrolysis of the p-nitrophenyl residue in the presence of poly C and G-containing oligonucleotide N-acetylhistidates. From the



Scheme 4.22. Schematic diagram showing the hydrolysis of I by II on III

References, pp. 483—508

hydrolysis rates observed the strength of interaction between the oligonucleotide derivatives and poly C was observed to decrease in the order $\text{dGpGpG} > \text{dGpGp} > \text{dGpC} > \text{dGpA} > \text{dGpT}$. These observations together with the specificities reported in the monomer approach (see above) suggest, that the prerequisite of proper alignment of the nucleotide blocks on the respective template can be fulfilled even with comparatively short chains (or even with monomers). It seems therefore not unlikely that template-directed synthesis especially with oligonucleotide blocks finally will become practical, although further work is necessary in order to improve the yields and/or to guarantee the formation of natural internucleotide bonds. In regard to the synthesis of specific base sequences, one more general drawback of template-directed synthesis (chemical or enzymic), namely the necessity of having proper templates and blocks available, should be kept in mind. Thus at least for the blocks to be connected and for the chains serving as templates conventional methods of synthesis seem unavoidable.

5. Formation of Internucleotide Linkages by Enzymic Reactions

Three main classes of enzymes have been exploited for synthetic reactions in polynucleotide chemistry. The first class, the polymerizing enzymes, can be subdivided into primer dependent and primer-template dependent enzyme species. Polynucleotide phosphorylase appears as the most prominent representative among the primer dependent enzymes, as numerous ribopolymers have been prepared by this enzyme already for the elucidation of the genetic code (193, 194, 197, 439). DNA dependent DNA polymerase I and DNA dependent RNA polymerase from *E. coli* represent the most frequently used primer-template dependent species and their application was essential, for example, for the preparation of polymers containing repeating di-, tri- and tetranucleotide (185, 186, 187, 197, 461). While polymerizing enzymes have long been established as valuable synthetic tools, the second class of enzymes represented by polynucleotide ligases has been introduced only more recently. Its use in connecting chemically synthesized segments has recently culminated in the total synthesis of two tRNA genes (2, 188). Finally ribonucleases, though generally regarded as cleaving agents, have been introduced as a third class of synthetic enzymes, especially for the synthesis of short oligoribonucleotides. This has been made possible by causing reversal of the cleaving reactions by adding a large excess of one of the cleavage products whereby the equilibrium is driven towards the side of internucleotide bond formation.

One general advantage of enzyme-catalyzed synthetic reactions consists in the specificity guaranteed by the respective enzymes. For this reason, blocking groups necessary for the protection of the various functional groups in organic chemical reactions are commonly not needed in enzymic reactions. On the other hand, only comparatively small quantities of synthetic material (often in the range of less than 1 mg) are accessible by enzymic reactions, unless huge amounts of enzymes which usually are costly or time consuming to prepare are applied. Only ribonuclease-catalyzed reactions could be performed on larger scale. While this limitation may be severe for physicochemical measurements, for which several milligrams and more are frequently necessary, studies in the biochemistry of polynucleotides or in molecular genetics quite often have been performed with quantities far below the milligram level of the respective polynucleotides (39, 130, 206, 366, 467).

5.1. Reactions Catalyzed by Polymerizing Enzymes

Reactions catalyzed by polymerizing enzymes can be subdivided into two classes. Enzymes of the primer dependent class add activated nucleotide units to the 3'-ends of short oligonucleotide primers to yield homopolymers or random copolymers according to Scheme 5.1. In



Scheme 5.1

the ribo series polynucleotide phosphorylase (175, 193, 194, 197, 439) (see also references given in Table 5.1) has been used extensively for this type of reaction, in which case ribonucleoside-5'-diphosphates serve as activated nucleotide units together with a dinucleoside monophosphate or longer ribooligonucleotides as primer. Inorganic phosphate is liberated at each step and in the presence of high concentrations of inorganic phosphate the reaction can be reversed towards phosphorolysis of ribopolynucleotides. In the deoxy series the enzyme most frequently used for the primer dependent synthesis of homopolymers or random copolymers is terminal deoxynucleotidyl transferase (30, 461), which utilizes deoxyribonucleoside triphosphates as substrates for the polymerization onto the 3'-end of a deoxytrinucleoside diphosphate as minimum primer. One equivalent of pyrophosphate is liberated for each nucleotide added.

References, pp. 483—508

In contrast to these primer dependent species in which the addition onto the primer is governed mainly by the availability of the respective di- or triphosphates in the reaction medium, a second class of polymerizing enzymes is dependent on the presence of a template/primer. DNA-dependent DNA polymerases and DNA-dependent RNA polymerases are well-known representatives of this class by which amount and sequence of the nucleotide units incorporated are governed by a DNA template. RNA-dependent RNA polymerases (as, for example, replicase induced by the phage Q β) (89, 327) have also long been known and more recently RNA dependent DNA polymerases, so called reverse transcriptases, have been discovered (102). Ribo- or deoxyribonucleoside triphosphates are the substrates for these enzymes. While a template (DNA or RNA) is compulsory for this class of enzymes, initiation of the polymerizing reaction without a primer is sometimes possible as in the cases of Q β replicase (89, 327) or of DNA-dependent RNA polymerases (186, 187, 206, 279). Using Q β replicase (89, 327, 377) or a combination of DNA-dependent DNA polymerase and polynucleotide ligase (111) *in vitro* synthesis of the total genomes of phage Q β or ϕ X174 respectively could be achieved.

Synthetic homopolymers, random copolymers and polymers containing repeating di-, tri- and tetranucleotides obtained by application of the various polymerizing enzymes have been reviewed in detail; these reviews include work published during the past 5 years (30, 186, 187, 197, 461). Only a list of polymers containing unusual nucleotides, base pairs or internucleotide linkage will therefore be given in this article (Table 5.1) as a number of polymers has been synthesized containing various modifications in the base, sugar or phosphate moieties.

One general limitation of template dependent polymerizing enzymes arises from the fact that base sequences of the products are entirely governed by the respective templates and that therefore synthesis of specific sequences other than the ones complementary to the template cannot be achieved. Although this limitation converts to a true advantage, where mere copying of already existing templates is desired (as for example in the reported *in vitro* synthesis of the whole genomes of phage Q β or ϕ X174), enzymic methods which would allow single step additions to a given primer, appear more attractive from a synthetic view point. For this purpose template independent enzymes such as terminal deoxynucleotidyl transferase and polynucleotide phosphorylase represent more favourable candidates, as probably any sequence (not only sequences programmed by templates) could be synthesized once a stepwise procedure is developed.

Table 5.1. Enzymically Prepared Oligo- and Polynucleotides Containing Unusual Bases, Unusual Base Pairs, Modified Nucleosides or Ribo-Deoxy-Internucleotide Linkages

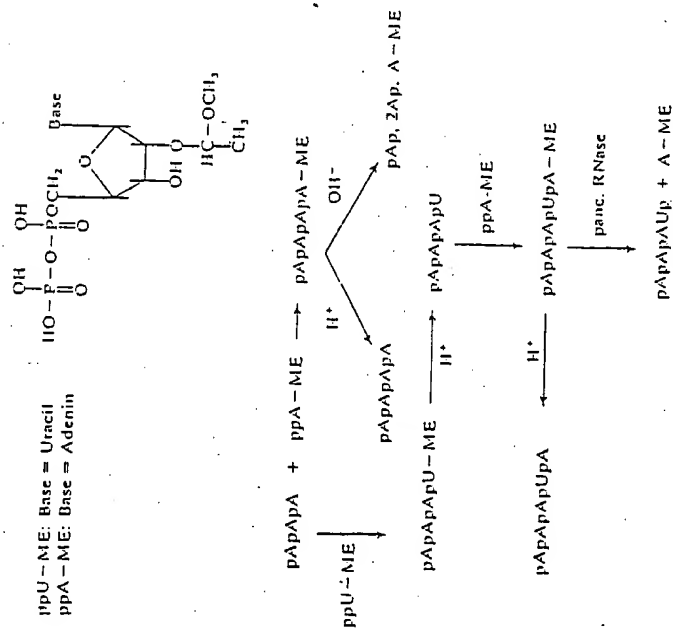
Polymer	Polymerizing enzyme	References
(dI-dC) _n	DNA dependent DNA polymerase I (E. coli)	(114, 115)
(dG-dT) _n	DNA dependent DNA polymerase I (E. coli)	(244)
(dA-d4thioT) _n	DNA dependent DNA polymerase I (E. coli)	(81, 240, 242, 243)
(dA-d2thioT) _n	DNA dependent DNA polymerase I (E. coli)	(241)
(dA-dC) · (dT-d6thioG) _n	DNA dependent DNA polymerase I (E. coli)	(17, 242)
(dA-d6thioG) _n · (dT-dC) _n	DNA dependent DNA polymerase I (E. coli)	(17, 242)
(dA-dC) · (dT-d6thioT) _n	DNA dependent DNA polymerase I (E. coli)	(17, 242)
(dA-dC) · (d4thioT-dG) _n	DNA dependent DNA polymerase I (E. coli)	(17, 242)
(dAcA) _n	Terminal deoxynucleotidyl transferase	(143)
(dAcG) _n	Terminal deoxynucleotidyl transferase	(143)
(dAcC) _n	Terminal deoxynucleotidyl transferase	(127)
(dibU) _n	Terminal deoxynucleotidyl transferase	(127)
(dU) _n	Terminal deoxynucleotidyl transferase	(127)
(dN) ₆ sepA, pA ₄	Terminal deoxynucleotidyl transferase	(208)
(dN) ₆ sepA, pA ₄	Terminal deoxynucleotidyl transferase	(208)
(dT) ₆ pN ₄ (N = A, C, G, U)	Terminal deoxynucleotidyl transferase	(360, 361)
(dT) ₆ pN ₄ (pM ₄) _n	Terminal deoxynucleotidyl transferase	(360, 361)
(dT) ₆ pA, pA ₄ (pdA) _n	Terminal deoxynucleotidyl transferase	(360)
(dT) ₆ pU _n	Terminal deoxynucleotidyl transferase	(208a)
(dT) ₆ pU, pU _n	Terminal deoxynucleotidyl transferase	(208a)
(dT) ₆ pA _n	Terminal deoxynucleotidyl transferase	(208a)
(dT) ₆ pA, pA _n	Terminal deoxynucleotidyl transferase	(208a)
(rA) ₆ - (dC) _n	Terminal deoxynucleotidyl transferase	(90, 91, 97)
ApApA	Polynucleotide phosphorylase	(178a)
ApApApA	Polynucleotide phosphorylase	(178a)
ApUpA	Polynucleotide phosphorylase	(178a)
(rA) _n -dA	Polynucleotide phosphorylase	(30)
(rA) _n -dA-dA	Polynucleotide phosphorylase	(30)
(rA) ₆ -T ₄ and (rA) ₆ -T ₆ -T ₆	Polynucleotide phosphorylase	(91, 92)
(rA) ₆ -T ₄ -(C ₆) _n	Terminal deoxynucleotidyl transferase	(91, 92)
(rA) ₆ -T ₄ -T ₄ -(C ₆) _n	Polynucleotide phosphorylase	(49, 51)
(rA, dA) _n	Polynucleotide phosphorylase	(448)
(r4thioU) _n	Polynucleotide phosphorylase	(412)
(r4-thiomethyl U) _n	Polynucleotide phosphorylase	(81, 372)
(r-phosphothio U) _n	Polynucleotide phosphorylase	(80, 81)
(r2thioC) _n	Polynucleotide phosphorylase	(373)
(r1) _n · (r2thioC) _n	Polynucleotide phosphorylase	(86, 373)

References, pp. 483-508

(rU, 4thioU) _n	Polynucleotide phosphorylase	(371)
(rU, 4thioT) _n	Polynucleotide phosphorylase	(371)
(rN ² , 5-dimethylU) _n	Polynucleotide phosphorylase	(368)
(r5-hydroxymethyl U) _n	Polynucleotide phosphorylase	(368)
(r5-methylU) _n	Polynucleotide phosphorylase	(368)
(r3-methyl T) _n	Polynucleotide phosphorylase	(29)
(2'-O-methyl A) _n	Polynucleotide phosphorylase	(413, 487)
(2'-O-methyl C) _n	Polynucleotide phosphorylase	(486)
(2'-O-methyl U) _n	Polynucleotide phosphorylase	(168, 224)
(rN ⁶ -etheno A) _n	Polynucleotide phosphorylase	(168)
(rN ⁴ -etheno C) _n	Polynucleotide phosphorylase	(148, 150)
(2'-chloro U) _n	Polynucleotide phosphorylase	(148, 150)
(2'-fluoro C) _n	Polynucleotide phosphorylase	(167)
(2'-amino U) _n	Polynucleotide phosphorylase	(149, 151)
(2'-amino C) _n	Polynucleotide phosphorylase	(151)
(2'-azido U) _n	Polynucleotide phosphorylase	(151)
(2'-azido C) _n	Polynucleotide phosphorylase	(442a, 444, 445)
(rΨ) _n	Polynucleotide phosphorylase	(110)
(r3-methyl U) _n	Polynucleotide phosphorylase	(445)
(r 5,6-dihydro U) _n	Polynucleotide phosphorylase	(443, 445)
(r 5,6-methylene U) _n	Polynucleotide phosphorylase	(443)
(r(A, G) _n · (U) _n	Polynucleotide phosphorylase	(8)
(r(I, U) _n · (rC) _n	Polynucleotide phosphorylase	(9)
(r(U, N ⁴ -hydroxy C) _n	Polynucleotide phosphorylase	(169)
(r(C, N ⁴ -hydroxy C) _n	Polynucleotide phosphorylase	(169)
(r3-methyl U) _n	Polynucleotide phosphorylase	(434)
(r7-methyl I) _n	Polynucleotide phosphorylase	(334)
(rN ² -acetyl C) _n	Polynucleotide phosphorylase	(334)
(rN ² -methyl G) _n	Polynucleotide phosphorylase	(335)
(rN ² -dimethyl G) _n	Polynucleotide phosphorylase	(335)
(r6-chloropurine) _n	Polynucleotide phosphorylase	(449)
(rC'A) _n	Polynucleotide phosphorylase	(125)
(rC'A) _n	Polynucleotide phosphorylase	(125)
(rH ⁶ A) _n	Polynucleotide phosphorylase	(125)
U-N-U _n	Polynucleotide phosphorylase	(374)
N-U _n	Polynucleotide phosphorylase	(374)
(N = 3-deazauridine, 4-deoxy-uridine, 3-deaza-4-deoxyuridine)	DNA-dependent RNA polymerase (E. coli)	(71, 81)
(rA-r4thioU) _n	DNA-dependent RNA polymerase (E. coli)	(77, 80, 81)
(r-phosphothio A-r-phosphothio U) _n		

In the ribo series polynucleotide phosphorylase has been successfully used for the stepwise synthesis of oligonucleotides containing specific sequences, when 2',3'-O-protected ribonucleoside diphosphates were

provided as substrates. According to Scheme 5.2 (179, 252) addition of such substrate results in a product containing a blocked 3'-terminus, whereby further addition is suppressed. Only after deblocking of the isolated product and addition of new substrate (again in the blocked



Scheme 5.2. Stepwise oligonucleotide synthesis with polynucleotide phosphorylase

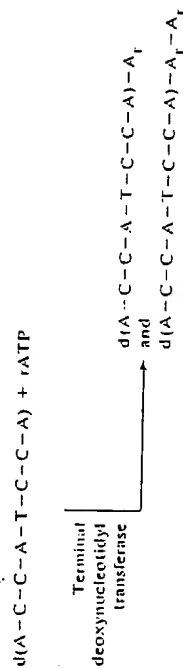
form) can subsequent addition take place. Repetition of this cycle using only one of the four protected ribonucleoside diphosphates as substrate at a time should lead – at least in principle – to any desired ribonucleotide sequence if the selection of the ribonucleoside diphosphate used as primer is properly adjusted to the sequence to be synthesized. The selection of the blocking group has to be adjusted to the following conditions: first, there should be no or minimum interference with the substrate binding site of the enzyme; secondly, the blocking group should maximally inhibit the primer function of the single addition product; thirdly the blocking group should be completely stable during the course of the reaction and should be removable under conditions that leave all other functional groups of the product intact. From the various

References, pp. 483—508

protecting groups tested the 2'(3')-O (α -methoxyethyl)-group (252) and the 2'(3')-O-isovaleryl group (179) seem to meet these requirements satisfactorily. In the case where the α -methoxyethyl group is used evidence has been presented that the 2'-O-derivatives of the respective ribonucleoside diphosphates are the substrates accepted by the enzyme leading to a monoaddition product with a blocking group at the 2'-hydroxyl function (18).

Although the 3'-hydroxyl function as such would therefore be free, further addition is apparently suppressed by steric hindrance of the adjacent 2'-O-protecting group which still remains. Extrapolating from this it seems likely that the 2'-O-derivatives represent the substrates also in the cases where the isovaleryl group was used for 2'(3') protection of ribonucleoside diphosphates (179); in this case, however, rapid equilibration between the 2'- and 3'-isomers will probably allow indirect utilization of the 3'-O-derivatives. Using this stepwise approach ribonucleotides of specific sequences up to the size of a pentanucleotide (252) or a heptanucleotide (180) have been prepared by two (252) or three (180) consecutive additions to the respective primers. Nearly quantitative yields seem to be possible in each step using the 2'(3')-derivatives of all four standard ribonucleoside diphosphates. Problems, may, however, arise 1) from the alkali treatment necessary for removal of the isovaleryl group (179), 2) from primer phosphorolysis induced by the inorganic phosphate liberated during the addition reaction (105), and 3) by transnucleotidation reactions (179). Although the results reported so far seem most encouraging, it remains to be seen whether further improvements are necessary in order to use the enzymic stepwise approach as a routine procedure. Also no reports are so far available on the maximum chain lengths accessible by these approaches.

Analogous attempts in the deoxy series apparently have not been successful. 3'-O-Acetylthymidine triphosphate seems not to be acceptable as a substrate for terminal deoxynucleotidyl transferase (127). This enzyme can, however, utilize ribonucleoside triphosphates (instead of deoxyribonucleoside triphosphates) for the limited addition of one or two nucleotide units to a given primer (208, 360, 361) (see Scheme 5.3).

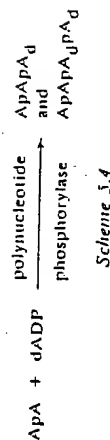


Scheme 5.3.

and this reaction offers some synthetic value for the specific extension of deoxyoligonucleotides. Thus, when the synthetic deoxyoligonucleotide d(A-C-C-A-T-C-C-A) was enzymically extended to the nonanucleotide d(A-C-C-A-T-C-C-A)-A, and decanucleotide d(A-C-C-A-T-C-C-A)-A, increased priming efficiency was observed with the latter in the presence of ϕ X174 DNA as template and DNA polymerase I (209). The two additions can also be performed in two successive steps with two different ribonucleoside triphosphates (R. ROYCHOUDHURY and H. KÖSSEL, unpublished observation). Based on this terminal addition reaction a method for the 3'-terminal labelling of oligodeoxynucleotides could be developed (208) which also allows partial sequences determination of oligodeoxynucleotides (379, 467).

The terminal addition of ribonucleotidyl residues to oligodeoxynucleotide primers by terminal deoxynucleotidyl transferase has also been used in order to make oligodeoxynucleotides more acceptable as primers for polynucleotide phosphorylase (FEIX and LINDER, unpublished). As evident from the fact that ribonucleoside triphosphates are accepted as substrates for a limited terminal addition reaction, the specificity requirement of the enzyme terminal deoxynucleotidyl transferase with respect to the sugar moiety of the substrates appears not to be rigorous. It seems therefore not unlikely that a masked deoxynucleoside triphosphate which mimics ribonucleoside triphosphate finally could be used as acceptable substrate for a stepwise terminal addition approach similar to the one already developed in the ribo series.

It is interesting to note that a terminal addition reaction reciprocal to the one observed for terminal transferase has been found for polynucleotide phosphorylase in the terminal addition of deoxynucleotidyl residues (instead of ribonucleotidyl residues) onto ribooligonucleotide primers (50, 178a) (Scheme 5.4). This reaction which is also limited



Scheme 5.4

to the addition of one or two nucleotidyl residues has been utilized in order to make oligoribonucleotides better acceptable as primers for terminal deoxynucleotidyl transferase (91, 92) though ribopolynucleotides and ribonucleotide terminated oligodeoxynucleotides also exhibit considerable priming activity in the presence of this enzyme (90, 360, 361).

The oligomer-initiated polymerization of unprotected deoxyribonucleotide units catalyzed by terminal deoxynucleotidyl transferase can

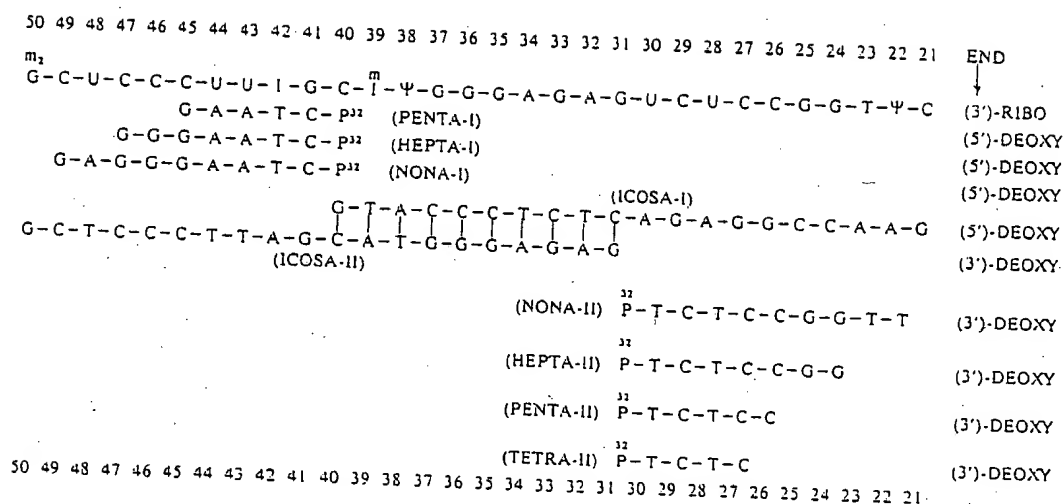
References, pp. 483—508

be limited to the formation of short polymers by using small ratios of deoxyribonucleoside 5'-triphosphates to primer (142, 349). The numbers of nucleotide residues added conform reasonably well with a Poisson distribution. When (T)₄ is used as primer with one molar equivalent of dCTP followed by another equivalent of dTTP, a product mixture of T₄C_mT_m(n, m = 0, 1, 2) is obtained in which only one member (n = 0; m = 2) is missing (142). Thus, successive stepwise addition (even of dinucleotide units) seems feasible by this method although it remains to be seen whether it can be applied to the synthesis of more complex sequences. As under comparable conditions the self-limiting polymerization of dGTP results in a very sharp product distribution, extension or even improvement of this method for the synthesis of G containing deoxypoligonucleotides seems possible. A somewhat similar principle has been applied to the synthesis of a three-section block copolymer of thymidylate, deoxyguanylate and deoxyadenylate by successive terminations of oligo dG and oligo dA blocks onto an oligo dT primer catalyzed by terminal deoxynucleotidyl transferase (349).

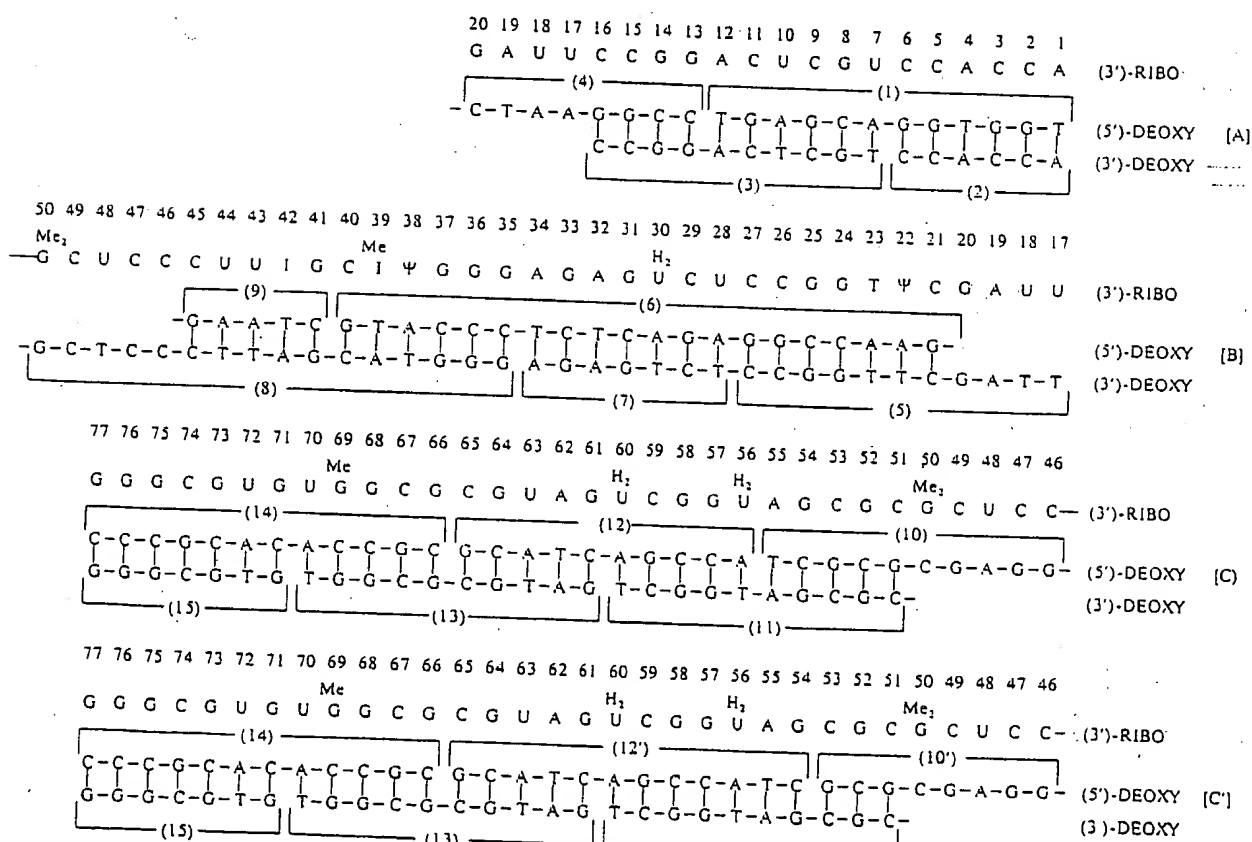
An enzyme capable of catalyzing primer dependent polymerization of deoxyribonucleoside 5'-diphosphates has recently been studied for single step addition onto d(pA)₄ (106). Although 4 to 8 fold molar excesses of one of the four deoxyribonucleoside 5'-diphosphates in the unprotected form were applied, single terminal addition products were obtained as the main products in reasonable yields. Thus, it seems not unlikely that this enzyme will also be useful in the stepwise synthesis of more complex oligodeoxynucleotides, although no such report has been published so far.

5.2. Reactions Catalyzed by Polynucleotide Ligases

Following the discovery of polynucleotide ligases in the mid sixties a strategy for the total synthesis of the gene coding for an alanine transfer RNA from yeast was immediately envisaged (2, 6, 185, 18 189). The two basic parts of this strategy consist first in synthesizing chemically oligodeoxynucleotide segments according to the known primary structure of the tRNA and second in joining these segments enzymically by polynucleotide ligase. In order to allow the enzymic joining reaction to occur each two segments to be joined must be held in adjacent position by means of base pairing with a third overlapping fragment (the "splint"). The 3'-hydroxyl group of one segment (the "acceptor") is thereby brought into juxtaposition of the 5'-terminal phosphate of the other (the "donor"). The splint thus, provides specific template guidance for the ligation process (Scheme 5.5).



Scheme 5.5. Ligation of oligodeoxynucleotide segments



Schen
to K:
Total synthesis of the structural gene for an alanine u
A *et al.* Ligation of the chemically synthesized oligodeoxynucleotide segments

Studies were carried out in order to determine the minimum lengths of the deoxyribonucleotide chains which polynucleotide ligases require to bring about the joining reaction (120, 121, 188). As these chain lengths turned out to be comparatively small (in one case even a tetranucleotide could be reacted as a "donor") the plan for the total synthesis of a tRNA gene was designed as follows: 1. Conventional chemical synthesis by the diester method of deoxypolynucleotide segments of chain lengths in the range of 8 to 12 units containing free 3'- and 5'-hydroxyl ends (see Table 4.1). These segments would represent the entire two strands of the intended DNA (Scheme 5.6) and would have to be selected such that those belonging to the complementary strands would allow an overlap of four to seven nucleotide units. 2. Enzymic phosphorylation of the 5'-ends by means of polynucleotide kinase (357), and 3. alignment of the appropriate segments to bihelical complexes and "sealing up" by polynucleotide ligase. The ligase catalyzed reactions generally can be performed with several components at a time.

Thus, for the synthesis of part B of the tRNA^{Ala} gene from yeast (Scheme 5.6), segments 7 and 9 in the 5'-phosphorylated form were simultaneously connected with fragments 8 and 6, respectively, which serve both as acceptor and splint segments at the same time. After this joining reaction was completed, segment 5 in the phosphorylated form was added to yield the entire part B with the two single stranded protruding ends ready for linkage with the complementary sticky ends of part A and C respectively (402). Synthesis of part A (400) and C (454) from the corresponding fragments was achieved in a similar multistep fashion. The large fragments finally were combined to the 77 base pairs containing bihelical duplex, by joining a preformed A + B product with part C or by joining a preformed B + C adduct with part A (39). More recently this technique has been successfully applied also to the synthesis of a gene corresponding to tyrosine suppressor tRNA from *E. coli* and its precursor comprising a total length of 126 base pairs (190).

There seems no doubt that this strategy would also prove successful for the synthesis of much larger genes or even of total genomes if the corresponding segments would be more easily accessible by chemical synthesis. As the average effort necessary for the chemical synthesis of a deca or dodecamer ranges between one quarter and one half of a "man-power-year", it becomes clear that chemical synthesis of the segments is the major rate-limiting part of the entire strategy at present and that therefore improved methods for the rapid chemical or enzymic synthesis of oligodeoxynucleotides are highly desirable.

References, pp. 483-503

One obvious prerequisite for the application of this strategy is that the base sequence of a gene to be synthesized has to be known in contrast to the approach, whereby genomes have been synthesized *in vitro* by mere copying of input templates in the presence of polymerizing enzymes (89, 111, 327). In the case of known RNA sequences the sequences of the respective genes can be deduced unambiguously by the base pairing rules as exemplified by the gene coding for tRNA^{Ala} from yeast (Scheme 5.6). However, due to the degeneracy of the genetic code unequivocal deduction of a nucleotide sequence from a known amino acid sequence can only be achieved to a limited extent (146, 288). For instance if the two amino acids methionine and tryptophan, for which only one codon exists, respectively, occur in neighbourhood to each other, oligonucleotide sequences containing no or very few ambiguities can be predicted (379, 380, 467). In general, however, more than one third of the nucleotide sequences derived from amino acid sequences are ambiguous and in many of these cases all four possible bases may occur in each ambiguous position. Although this offers the advantage, that the synthetic routes to the gene fragments to be prepared can partly be adjusted to maximum simplicity (146, 288), the risk of selecting by chance rare codons has to be kept in mind. As it is hoped that synthetic genes finally will be introduced and transcribed in living cells, selection of rare codons may then forbid effective expression of synthetic genes. The only way of avoiding this problem seems to consist in sequence analysis of the respective mRNAs or DNAs prior to gene synthesis. Sequence analysis will also be a prerequisite to the synthesis of untranslated or untranslated DNA sequences such as intercistronic regions or regulatory elements such as operator or promotor regions.

In order to obtain maximum yields and/or to avoid certain undesired deviations in the joining reactions catalyzed by polynucleotide ligase, the reaction components and conditions have to be selected carefully. Thus, effective joining of segment 5 in part B of the tRNA^{Ala} gene (Scheme 5.6) requires raising of the temperature to 25°C, whereas a temperature of 15°C is sufficient for the joining of segments 6, 7, 8 and 9 to each other (188, 402). This temperature dependency probably reflects internal secondary structures of the oligonucleotide segments, which interfere with the annealing of the segment to be joined.

An instructive deviation from the expected joining reaction was observed when an attempt was made to react the partial duplex obtained from segments 1, 2 and 3 (part A of the tRNA^{Ala} gene, Scheme 5.6) with segment 4 (188, 400). When the 5'-terminus of segment (3+2) was phosphorylated, the product formed was a dimer of the starting duplex (comprising two copies of the segments 1, 2 and 3). This

dimerization obviously was due to the self-complementary nature of the protruding C-C-G single-stranded end of the duplex (1, 2, 3). Joining with the segment 4 was only observed when the 5'-terminal phosphate of the (3+2) segment was absent as this phosphate is required for dimerization.

In order to prevent undesired joining reactions protection of 5'-terminal phosphate groups on the acceptor or splint molecules by alkylation groups has been proposed (129). Interference of such unphysiological groups with the joining reaction apparently is not encountered as they are positioned far enough from the joining center. This modification may therefore indeed prove helpful during further synthetic work in order to prevent alternate wrong joining reactions. Besides self-complementarity of segments to be reacted, infidelity of the joining reaction itself may create additional possible problems. Thus, when pT₁₁-C was reacted (as donor and acceptor molecule) on poly dA as template in the presence of T₄-induced ligase, head to tail joining to the mismatched C-residues was observed (446).

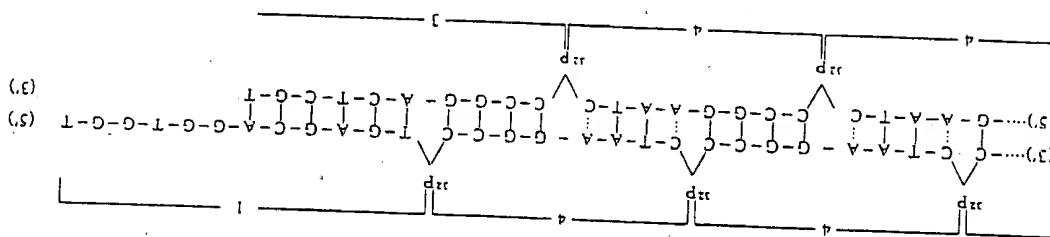
A-C base pairs have also been found acceptable in an oligomerization reaction observed with segment 4 of the tRNA^{Ala} gene (see Scheme 5.7) (400). Whether other nonclassical base pairs are acceptable or whether more than one mismatched base pair can be tolerated near the joining site remains to be seen. Another interesting type of deviation from the normal joining reactions was observed in the joining of DNA duplexes at completely base paired ends (399, 404). It is difficult to evaluate this type of deviation for synthetic purposes as it may constitute an undesired side reaction in one case or in other cases a synthetic aid.

Ribooligonucleotides can also be reacted as substrates of polynucleotide ligase (195, 367*a*). Thus, head-to-tail joining of ribooligonucleotides in the presence of poly dT was observed. A reciprocal substrate situation (ribo-template, deoxyligonucleotides to be joined) seems also acceptable as evident from the joining reaction observed with oligo dT on a poly rA template. SANO and FEIX have recently demonstrated that – in contrast to earlier results (195) – all-ribo substrates (with the exception of oligo rA on a poly rU template) are also acceptable for polynucleotide ligase catalyzed reactions (367*a*). There is no doubt that these “deviations” toward ribo-substrates are widening the possible usefulness of ligases as synthetic tools.

The ligation technique has recently been extended to the joining of a chemically synthesized short oligomer onto a naturally occurring d(PA-G-G-T-C-G-C-G-C-C) molecule of high molecular weight. Thus, the synthetic dodecamer d(PA-G-G-T-C-G-C-G-C-C) was annealed and covalently joined to lambda phage DNA in the presence of T₄-ligase (130). This is the

References, pp. 483--508

Scheme 5.7. Ligation of partially mismatched oligodeoxynucleotide segments

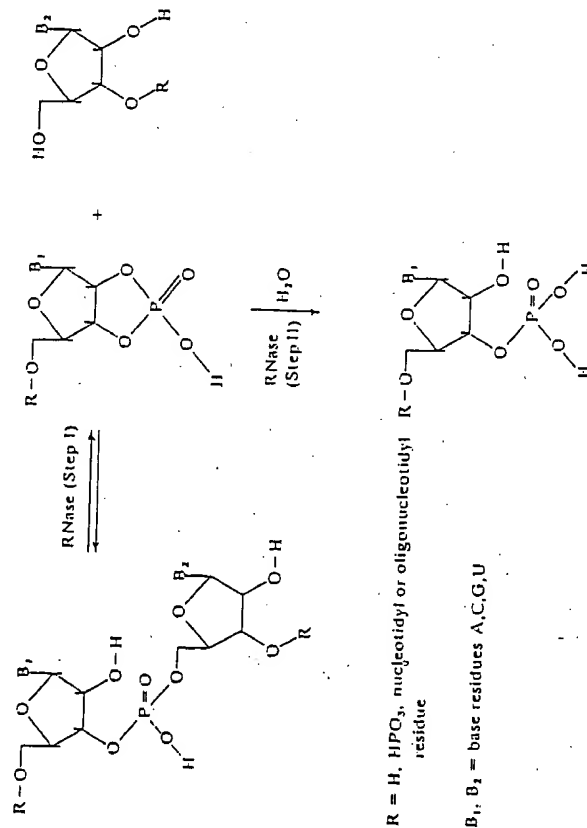


first time that a chemically synthesized oligonucleotide has been covalently linked to a naturally occurring phage DNA and this approach may be of general importance for future insertion of synthetic genes into living cells by using phage DNA as a "vehicle".

Polynucleotide ligase has allowed the preparation of a circular bihelical DNA containing repeating dinucleotide sequences (328).

5.3. Synthetic Reactions Catalyzed by Ribonucleases

This approach which is restricted to internucleotide bond formation in the ribo series is based on the following observations. During the breakdown of RNA catalyzed by ribonucleases transesterification to the respective 2',3'-cyclophosphates in many cases occurs as the first reaction step (Scheme 5.8). The second step, hydrolysis of the cyclic phosphate, then leads to the final products, the 3'-monophosphate derivatives. Though the latter step is virtually irreversible, in many



Scheme 5.8. Ribonuclease catalyzed formation of internucleotide linkages in the ribo series
References, pp. 483-508

instances conditions could be found under which it proceeds at a much slower rate than the transesterification step. As the transesterification at the same time constitutes a reversible type of reaction, the equilibrium can be shifted from the cyclic phosphate towards the side of internucleotide bond formation merely by application of high concentration of the 5'-hydroxyl carrying component. This approach already used in earlier work with ribonuclease A from bovine pancreas and with ribonuclease T₁ from *Aspergillus oryzae* has led to the stepwise synthesis of dinucleoside monophosphates and of trinucleoside diphosphates (21, 119, 165, 272, 388). More recent investigations have demonstrated (a) that several other ribonucleases can serve as synthetic tools (b); that the ribonuclease approach is also feasible for comparatively large scale synthesis (up to gram scale) (c); that coupling of preformed oligonucleotide blocks is possible and (d), that nucleotides containing altered base residues in some cases are also acceptable for the ribonuclease catalyzed reactions.

Besides ribonuclease A and ribonuclease T₁ which specifically catalyze the transesterification reactions from 2',3'-pyrimidine cyclophosphates and from 2',3'-guanosine cyclophosphates, respectively, ribonuclease N₁ from *Neurospora crassa* (201, 199), ribonuclease U₂ from *Ustilago sphaerogena* (165, 200, 450), and nonspecific ribonucleases from *Bacillus subtilis* (365) and *Aspergillus clavatus* (16, 485) have now been introduced as synthetic enzymes. Ribonuclease N₁ - like ribonuclease T₁ - specifically catalyzes transesterification reactions from 2',3'-guanosine cyclophosphate containing nucleotides onto a 5'-hydroxyl containing acceptor component (201, 199). The yields obtained with both the G specific enzymes (see Table 5.3) are also strongly influenced by the nature of the acceptor molecules with cytidine serving as most efficient acceptor followed by uridine and adenosine. The same order is apparent, where 2',3'-cycloguanosine phosphate has been reacted with dinucleoside monophosphates and where 2',3'-cycloguanosine phosphate terminated dinucleotides have been linked to nucleosides (see Table 5.3). Ribonuclease U₂ exhibits a broader specificity in respect to the 2',3'-cyclophosphate moiety, as it catalyzes transesterification from both the 2',3'-purine cyclophosphates (165, 200, 450; Table 5.3). The specificity in respect to the nucleoside acceptors seems to be similar to the one observed with the ribonucleases T₁ and N₁. The two nonspecific ribonucleases from *Bacillus subtilis* (365) and from *Aspergillus clavatus* (16, 485) have been studied for the synthesis of almost all possible dinucleoside monophosphates, which are obtained in satisfactory yields (Table 5.3). It is noteworthy that the enzyme from *Aspergillus clavatus* has also been used successfully for large scale reactions (16) with several grams of each component.

Table 5.3. Oligoribonucleotides Synthesized by Ribonuclease Catalyzed Reactions

Reaction type	RNase type	Products (% yield)	References
ApC>p+U	pancr. RNase	ApCpU (12)	(298)
ApU>p+Up	pancr. RNase	ApUpUp (11)	(298)
Py>p+Pu	pancr. RNase	PyPu (7-15)	(441)
OpC>+C	pancr. RNase	UpCpC (40)*	(182)
PupPy>p+N	pancr. RNase	PupPyN (4-12)	(181)
U>p+U	pancr. RNase	(Up) _n U	(103)
	pancr. RNase	(Up) _n >p	(103)
U>p+N	pancr. RNase	(Up) _n N	(103)
	pancr. RNase	(Up) _n	(103)
U>p+PupPu	pancr. RNase	(Up) _n U>p	(103)
	pancr. RNase	UpPupPu (4-18)	(104)
G>p+N	polymer bound		
	T ₁ -RNase	GpC (20, 66)	(272, 388)
	T ₁ -RNase	GpU (13, 62)	(272, 388)
	T ₁ -RNase	GpI (10, 32)	(272, 388)
	T ₁ -RNase	GpA (5, 4)	(388)
	T ₁ -RNase	GpX (5)	(374)
	T ₁ -RNase	GpU (12)	(165)
	RNase N ₁	GpC (44, 79**)	(199)
	RNase N ₁	GpU (12, 27)	(109)
	RNase N ₁	GpA (4)	(199)
	RNase N ₁	GpU (26)	(165)
	Actinomycin RNase	GpC (40)	(442)
I>p+C	RNase N ₁	IpC (22)	(201)
Pu>p+N	RNase U ₂	ApC (35, 67***)	(200)
	RNase U ₂	ApU (22, 19, 48***)	(200, 450)
	RNase U ₂	ApI (10)	(200)
	RNase U ₂	ApA (6)	(200)
	RNase U ₂	AphioU (30)	(165)
G>p+Up	T ₁ -RNase	GpUp (20)	(272)
A>p+Np	RNase U ₂	ApCp (5)	(200)
	T ₁ -RNase	ApCp (1)	(200)
G>p+NpM	T ₁ -RNase	GpCpC (20, 27, 40)	(119, 272, 388)
	T ₁ -RNase	GpCpU (14, 23)	(119, 388)
	T ₁ -RNase	GpCpA (12, 14)	(119, 388)
	T ₁ -RNase	GpCpG (3.5, 8)	(119, 388)
	T ₁ -RNase	GpUpC (7, 19, 37)	(119, 272, 388)
	T ₁ -RNase	GpApC (8, 25)	(24, 119)
	T ₁ -RNase	IpCpC (20)	(119)

* Ū symbolizes a uridine residue modified by addition of water soluble carbodiimide.

** Uchida, unpublished.

*** Uchida and Funayama, unpublished.

References, pp. 483-508

G>p+ApApC	T ₁ -RNase	IpCpU (19)	(119)
NpG>p+N	T ₁ -RNase	IpCpA (15)	(119)
	RNase N ₁	IpCpG (7)	(119)
	T ₁ -RNase	GpApApC (7)	(201)
	T ₁ -RNase	CpGpC (8)	(119)
	T ₁ -RNase	CpGpU (8, 10)	(119, 272)
	T ₁ -RNase	CpGpA (3)	(119)
	T ₁ -RNase	UpGpC (6)	(119)
	T ₁ -RNase	UpGpU (3, 38)	(119, 272)
ApUpG>p+N	T ₁ -RNase	ApGpU (53)	(272)
	T ₁ -RNase	ApUpGpC (32)	(272)
	T ₁ -RNase	ApUpGpU (37)	(272)
ApUpG>p+Np	T ₁ -RNase	ApUpGpA (30)	(272)
	T ₁ -RNase	ApUpGpCp (15)	(272)
	T ₁ -RNase	ApUpGpUp (15)	(272)
	T ₁ -RNase	ApUpGpAp (5)	(272)
ApUpG>p+oligo(A) ₂₋₃	T ₁ -RNase	ApUpGpApAp (11)	(272)
	T ₁ -RNase	ApUpGpApApAp (8)	(272)
	T ₁ -RNase	ApUpGpApApApAp (5)	(272)
G>p polymerization	T ₁ -RNase	ApUpGpApApApAp (3)	(272)
	T ₁ -RNase	(Gp) _n n=2 (14)	(139)
	T ₁ -RNase	n=3 (6)	(139)
	RNase N ₁	n=4 (10)	(139)
	RNase N ₁	n=2 (22)	(199)
	RNase N ₁	n=3 (18)	(199)
	RNase N ₁	n=4 (7)	(199)
ApG>p polymerization	RNase N ₁	(ApGp) _n n=2 (33)	(201)
	RNase N ₁	n=3 (7)	(201)
A>p polymerization	RNase U ₂	(Ap) _n n=2 (17)	(200)
	RNase U ₂	n=3 (5)	(200)
N>p+Py	A. clavatus RNase	NpPy (8-40)	(200)
N>p+Py	B. subtilis RNase	NpPy (20-75)	(16, 485)
			(365)

The coupling reaction could be extended to the joining of preformed oligonucleotide blocks (119, 201, 272, 388). As summarized in Table 5.3, the cyclophosphate bearing components as well as the acceptor components can constitute longer oligonucleotide chains. The maximum chain length synthesized by the ribonuclease approach seems to be the octanucleotide A-U-G(-A)₃ from the blocks A-U-G> and A(pA)₃. The coupling of oligomeric components is also evident from the oligomerization reactions observed with 2'-3'-cyclo AMP, 2'-3'-cyclo GMP and ApG> in the presence of ribonuclease U₂ and ribonuclease N₁ respectively (201, 199).

Oligomerization of 2',3'-cyclo GMP in the presence of ribonuclease T₁ has already been studied earlier (139). More recently, however, the product of this reaction has become a matter of controversy as it was demonstrated (336, 337, 338) that the products formed at room

temperature almost exclusively contain 2'—5'-linkages (as evident from resistance against T_1 -ribonuclease and from degradation to 2'-GMP). This is in contradiction to all other reports on ribonuclease T_1 catalyzed synthesis (119, 139, 165, 272, 388) where the internucleotide bonds of the products formed at 4°C proved to be entirely susceptible to enzymic degradation, which evidences natural 3'—5'-linkages. Whether this discrepancy is due to a temperature effect or to contaminants in the enzyme preparations used remains open. Even if these controversial findings remain a single case, it nevertheless underlines the necessity of carefully characterizing the products obtained by the ribonuclease approach.

The yields obtained in ribonuclease catalyzed synthetic reactions range between 5 and 30% for short chains (see Table 5.3). In the special case of GpC yields up to 66% and even higher have been reported. Though better yields are generally obtained by chemical methods especially for ribooligonucleotides of medium and higher chain length, the enzymic approach clearly offers the following two advantages: (a) no protecting groups are required either for the 2'-OH functions or for the amino functions on the bases, (b) reactions are carried out entirely in aqueous medium, thereby avoiding the problem of dissolving oligonucleotides in organic solvents such as anhydrous pyridine. On the other hand, the ribonuclease approach is severely limited by the fact that cleavage of the internucleotide bonds already present can only be avoided in the case of base specific ribonucleases (such as T_1) when the respective internucleotide bonds are not present in both the starting components; thus ribonuclease T_1 can only be used for the joining of blocks which do not contain any internal or 5'-terminal guanosine residues.

Synthesis of higher oligonucleotides on larger scale may also be limited because it is difficult to denature or completely remove ribonucleases, which may interfere in subsequent reaction steps or during the workup. In order to eliminate this problem ribonuclease A fixed to solid supports such as CM-cellulose or to maleic anhydride copolymers has been used for the synthesis of the three terminator codons UAA, UAG and UGA from 2',3'-cyclo UMP and the respective dinucleoside monophosphates (104). The three codons could be isolated in 4—18% yield. This comparatively low yield is, however, fully counterbalanced (a) by the rapidity of the method, (b) by the nearly quantitative recovery of the unutilized dinucleoside monophosphates and (c) by the possibility to use the CM cellulose bound ribonuclease repeatedly in several reaction steps without loss of activity.

Introduction of nucleotides containing modified base moieties into oligonucleotides by ribonuclease catalyzed reactions has been reported

References, pp. 483—508

in several cases. Inosine, Xanthosine, and ApI can serve as acceptor molecules in reactions catalyzed by T_1 or U_2 ribonuclease (119, 272, 388). 2',3'-Cyclophosphates derived from inosine, 8-azaguanosine and xanthosine have been used as activated mononucleotides in the presence of ribonuclease T_1 (119) and N_1 (199, 201). 2',3'-Cyclo GMP can be reacted with 4-thiouridine in the presence of ribonuclease T_1 or N_1 (165) and transfer of 2',3'-cyclo AMP to 4-thiouridine can also be achieved in the presence of ribonuclease U_2 . Codons containing in the wobble position the modified nucleosides 4-deoxyuridine, 3-deazauridine and 3-deaza-4-deoxyuridine have been synthesized by using ribonuclease A (103).

The same enzyme has been used for synthetic reactions involving 5'-O-methylphosphoryl-uridine 2',3'-cyclic phosphate as a class of substrates modified at the 5'-terminal phosphate by an unnatural substituent (21).

References

1. AGARWAL, K. L., and M. M. DHAR: The use of 2,3'-anhydronucleosides in the synthesis of the internucleotide bond. *Tetrahedron Letters* 2451 (1965).
2. AGARWAL, K. L., H. BÜCHLI, M. H. CARUTHERS, N. GUPTA, H. G. KHORANA, K. KLEPPE, E. OHTSUKA, U. L. RAJBHANDARY, J. H. VAN DE SANDE, V. SGARANELLA, H. WENGER, and T. YAMADA: Total synthesis of the gene for an alanine transfer ribonucleic acid from yeast. *Nature* 227, 27 (1970).
3. AGARWAL, K. L., A. YAMAZAKI, and H. G. KHORANA: Studies on polynucleotides. XCVIII. A convenient and general method for the preparation of protected dideoxyribonucleotides containing 5'-phosphate end groups. *J. Amer. Chem. Soc.* 93, 2754 (1971).
4. AGARWAL, K. L., and H. G. KHORANA: Studies on Polynucleotides. CII. The Use of Aromatic Isocyanates for Selective Blocking of the Terminal 3'-Hydroxyl Group in Protected Dideoxyribonucleotides. *J. Amer. Chem. Soc.* 94, 3578 (1972).
5. AGARWAL, K. L., A. KUMAR, and H. G. KHORANA: Studies on Polynucleotides. CIX. Total synthesis of the structural gene for an alanine transfer ribonucleic acid from yeast. Synthesis of a dodecadeoxynucleotide and a decadeoxynucleotide corresponding to the nucleotide sequence 46 to 65. *J. Mol. Biol.* 72, 351 (1972).
6. AGARWAL, K. L., A. YAMAZAKI, P. J. CASITION, and H. G. KHORANA: *Chemisch. Synthese von Polynucleotiden*. *Angew. Chem.* 84, 489 (1972).
7. AGARWAL, K. L., M. FRIDKIN, E. JAY, and H. G. KHORANA: Deoxynucleotide synthesis using a new phosphate protecting group. *J. Amer. Chem. Soc.* 95, 2070 (1973).
8. AKUTSU, H., and M. TSUBOI: Structure of polynucleotide complex with non-complementary nucleosides. I. Poly A, G + poly U. *Bull. Chem. Soc. Japan* 43, 3391 (1970).
9. ———: Structure of polynucleotide complex with non-complementary nucleosides. II. Poly I, U + poly C. *Bull. Chem. Soc. Japan* 44, 20 (1971).
10. AMAGAEVA, A. A., A. M. YURKEVITCH, I. P. RUDAKOVA, L. V. KHRISTENKO, I. M. KUSTANOVICH, and N. A. PREORASHENSKI: 5'-O- γ -Tolylsulfonyladenine derivatives. *Rhim. Priro. Soedin.* 4, 304 (1968). *Chem. Abstr.* 70, 115471s (1969).
11. ASAI, M., M. MIYAKI, and B. SHIMIZU: Synthetic nucleotides. II. A direct synthetic method for ribonucleotides. *Chem. Pharm. Bull. (Tokyo)* 15, 1856 (1967).

12. BADASHKEVA, A. G., G. N. KADASHIEVA, D. G. KNORRE, G. G. SHAMOVSKIY, and T. N. SHUBINA: Condensation of trioxynadylate by means of a water-soluble carbodiimide in the presence of polyuridylic acid. Dokl. Akad. Nauk SSSR 206, 870 (1972).
13. BÄHR, W., H. SOMMER, and K. H. SCHEIT: Synthesis and properties of oligodeoxy-4-thiothymidylic acid. Biochim. Biophys. Acta 287, 427 (1972).
14. BAKER, D. R., P. M. TANNA, and G. D. F. JACKSON: Non-classical antimetabolites. XXII. Simulation of 5'-phosphoribosyl binding. IV. Attempted simulation with nucleoside-5'-carbamates. J. Pharm. Sci. 54, 987 (1965).
15. RAZULAY, I., J. L. SUSSMAN, and Y. LAPIDOT: Further studies on the chromatograph behaviour of dinucleoside monophosphates. J. Chromatog. 79, 139 (1973).
16. BAUER, S., R. LAMED, and Y. LAPIDOT: Large scale synthesis of dinucleoside monophosphates catalyzed by ribonuclease from *Aspergillus clavatus*. Biotechnol. Bioeng. XIV, 861 (1972).
17. BEIKIRCH, H. H., and A. G. LEZIUS: Double-stranded Polydeoxyribonucleotides containing 6-Thiothiopyranosine and 6-Thiothiopyranosine. Poly[d(A-C).d(T-S⁶G)], Poly[d(A-S⁶G).d(T-C)], and Poly[d(A-C).d(T-S⁶T)]. Eur. J. Biochem. 27, 381 (1972).
18. BENNETT, G. N., J. K. MACKAY, J. L. WIEBERS, and P. T. GILIANI: 2-(O- α -methoxyethyl)-nucleoside-5'-diphosphates as Single Addition Substrates in the Synthesis of Specific Oligoribonucleotides with Polynucleotide Phosphorylase. Biochemistry 12, 3956 (1973).
19. BERGER, H.: Solvolysis and phosphorylating activity of polyphosphate esters. Z. Naturforsch. B 26, 694 (1971).
20. BERLIN, YU. A., O. G. CHAKIMAKHICHEVA, V. A. EFIMOV, M. N. KOLOSOV, and V. G. KOROBKO: Arenesulfonyl imidazolates, new reagents for polynucleotide synthesis. Tetrahedron Letters 1353 (1973).
21. BERNFIELD, M. R., and F. M. ROTTMAN: Ribonuclease and oligoribonucleotide synthesis. III. Oligonucleotide synthesis with 5'-substituted uridine 2',3'-cyclic phosphates. J. Biol. Chem. 242, 4134 (1967).
22. BLACKBURN, G. M., M. J. BROWN, and M. R. HARRIS: Nucleic acid studies on insoluble polymer supports. Chem. Commun. 611 (1966).
23. BLACKBURN, G. M., M. J. BROWN, and M. R. HARRIS: Synthetic studies of nucleic acids on polymer supports. Part I. Oligodeoxyribonucleotide synthesis on an insoluble polymer support. J. Chem. Soc. 2438 (1967).
24. BLACKBURN, G. M., M. J. BROWN, M. R. HARRIS, and D. SHIRE: Synthetic studies of nucleic acids on polymer supports. Part II. Mechanisms of phosphorylation with carbodi-imides and arenesulphonyl chlorides. J. Chem. Soc. 676 (1969).
25. BLANK, H. U., D. FRAUNE, A. MYLES, and W. PLEIDERER: Nucleosides. IV. Tritylation and benzoylation of adenosine derivatives. Liebig's Ann. Chem. 742, 34 (1970).
26. BLANK, H. U., and W. PLEIDERER: Nucleosides I. Syntheses of O'-benzyl derivatives of uridine. Liebig's Ann. Chem. 742, 1 (1970).
27. — — — Nucleosides. II. Tritylations and benzylations of cytidine derivatives. Liebig's Ann. Chem. 742, 16 (1970).
28. — — — Nucleosides. III. New mild method for the selective N⁶-acylation of cytidine. Liebig's Ann. Chem. 742, 29 (1970).
29. BOBST, A. M., P. A. CERUTTI, and F. ROTTMAN: The structure of poly 2'-O-methyladenylic acid at acidic and neutral pH. J. Amer. Chem. Soc. 91, 1246 (1969).
30. BOLLUM, F. J.: Terminal deoxynucleotidyl transferase. In: The Enzymes, X (P. D. BOYER, ed.), 145 (1974).
31. BOOM, J. H. VAN, P. M. J. BURGERS, G. P. OWEN, C. B. REESE, and R. SAFFHILL: Approaches to oligoribonucleotide synthesis via phosphotriester intermediates. Chem. Commun. 869 (1971).
32. BOOM, J. H. VAN, P. VAN DEURSEN, J. MEEUWSE, and C. B. REESE: Two sulfur-containing protecting groups for alcoholic hydrogen functions. J. Chem. Soc., Chem. Commun. 766 (1972).
33. BRANDSTETTER, F., H. SCHOTT, and E. BAYER: Liquid-phase-Synthese von Nucleotiden. Tetrahedron Letters 2997 (1973).
34. BREITENBACH, J. W., and O. F. OLAR: Über die proliferierende Polymerisation in Vernetzungssystemen und dynamisch-kinetische Messungen an Popcorn-Polymeren. Chimia 22, 157 (1968).
35. BROWN, D. M.: Phosphorylation. Advances Org. Chemistry 3, 75 (1963).
36. BRUNNGRABER, E. F., and E. CHARGAFF: Transferase from *Escherichia coli* effecting low-energy phosphate transfer to nucleosides and nucleotides. Proc. Natl. Acad. Sci. US 67, 107 (1970).
37. BÜCHI, H., and H. G. KRIORANA: Studies on Polynucleotides. CV. Total synthesis of the structural gene for an alanine transfer ribonucleic acid from yeast. Chemical synthesis of an icosa-deoxyribonucleotide corresponding to the nucleotide sequence 31 to 50. J. Mol. Biol. 72, 251 (1972).
38. CARUTHERS, M. H., and H. G. KRIORANA: Studies on Polynucleotides. CXI. To synthesis of the structural gene for an alanine transfer ribonucleic acid from yeast. Synthesis of a dodecadeoxyribonucleotide and a heptadeoxyribonucleotide corresponding to the nucleotide sequence 66 to 77. J. Mol. Biol. 72, 407 (1972).
39. CARUTHERS, M. H., K. KLEPPE, J. H. VAN DE SANDE, V. SGARAMELLA, K. L. AGARWAL, H. BÜCHI, N. K. GUPTA, A. KUNAR, E. OHTSUKA, U. L. RAJ BHANDARY, T. TERAO, H. WEBER, T. YAMADA, and H. G. KRIORANA: Studies on Polynucleotides. CXV. Total synthesis of the structural gene for an alanine transfer RNA from yeast. Enzymic joining to form the total DNA duplex. J. Mol. Biol. 72, 475 (1972).
40. CARUTHERS, M. H., J. H. VAN DE SANDE, and H. G. KRIORANA: Studies on Polynucleotides. CX. Total synthesis of three decadeoxyribonucleotides corresponding to the nucleotide sequence 51 to 70. J. Mol. Biol. 72, 375 (1972).
41. CASHION, P. J., M. FRIDKIN, K. L. AGARWAL, E. JAY, and H. G. KRIORANA: Studies on Polynucleotides. CXII. The use of trityl- and α -naphthylcarbamoyl cellulose derivatives in oligonucleotide synthesis. Biochemistry 12, 1985 (1973).
42. CATLIN, J. C., and F. CRAMER: Deoxyoligonucleotide synthesis via the triester method. J. Org. Chem. 38, 245 (1973).
43. CEDERGREN, R. J., B. LARUE, and P. LAPORTE: The acylation of ribonucleotides with benzoic and acetic anhydrides in aqueous solutions. Can. J. Biochem. 49, 730 (1971).
44. CHAPMAN, T. M., and D. G. KLEID: Oligonucleotide synthesis on polar polymer supports: The use of a polypeptide support. J. Chem. Soc. D. Chem. Commun. 193 (1973).
45. CHARGAFF, E., and J. N. DAVIDSON: The nucleic acids. Vol. 1, 2 (1955); Vol. 3. New York: London: Academic Press, 1960.
46. CHEN, H.-C., L. C. CRAIG, and E. STONER: On the removal of the residual carboxylic acid groups from cellulosic membranes and Sephadex. Biochem. 11, 3559 (1972).
47. CHLAPPEK, S., J. ZEMLECKA, and V. GUT: 5-chloro-8-hydroxyquinoline (chloroxine) esters of carboxylic acids — selective reagents for acylation of nucleoside and nucleotide aminoacyl derivatives. Biochem. Biophys. Res. Commun. 35, 306 (1969).
48. CHONG, K. J., and T. HATA: *p*-Nitrophenyl phosphates as phosphorylating reagents for alcohols. Bull. Chem. Soc. Jap. 44, 2741 (1971).
49. CHOU, J. Y., and M. F. SINGER: Synthesis of a copolymer containing adenylic and deoxyadenylic acid residues with polynucleotide phosphorylase. Biochem. Biophys. Res. Commun. 42, 306 (1971).
50. — — — Deoxyadenosine diphosphate as a substrate and inhibitor of polynucleotide phosphorylase of *Micrococcus luteus*. I. Deoxyadenosine diphosphate as a substrate for

51. CHOU, J. Y., and M. F. SINGER: Deoxyadenosine diphosphate as a substrate and inhibitor of polynucleotide phosphorylase of *Micrococcus luteus*. III. Copolymerization of adenosine diphosphate and deoxyadenosine diphosphate. *J. Biol. Chem.* 246, 7505 (1971).
52. CRISTENSEN, L. F., and A. D. BROOM: Specific chemical synthesis of ribonucleoside O-Benzyl ethers. *J. Org. Chem.* 37, 3398 (1972).
53. CLARK, V. M., D. W. HUTCHINSON, A. J. KIRBY, and S. G. WARREN: Phosphorylierungsmittel — Bauprinzip und Reaktionsweise. *Angew. Chem.* 76, 704 (1964).
54. COOK, A. F.: The use of β , β -tribromoethylchloroformate for the protection of nucleoside hydroxyl groups. *J. Org. Chemistry* 33, 3589 (1968).
55. COOK, A. F., M. J. HOLMAN, and A. L. NUSSBAUM: Nucleoside S-alkyl phosphorothioates. II. Preparation and chemical and enzymatic properties. *J. Amer. Chem. Soc.* 91, 1522 (1969).
56. — — — Nucleoside S-alkyl phosphorothioates. III. Application to oligonucleotide synthesis. *J. Amer. Chem. Soc.* 91, 6479 (1969).
57. COOK, A. F., and D. T. MATCROCK: Use of chloroacetic anhydride for the protection of nucleoside hydroxyl groups. *J. Org. Chem.* 35, 1940 (1970).
58. COOK, A. F., E. P. HEIMER, M. J. HOLMAN, D. T. MATCROCK, and A. L. NUSSBAUM: Nucleoside S-alkyl phosphorothioates. V. Synthesis of a deca-deoxyribonucleotide. *J. Amer. Chem. Soc.* 95, 1334 (1972).
59. COOPERMAN, B. S., G. J. LLOYD, and C.-M. HSU: Reactivity of phosphorylimidazole, an analog of known phosphorylated enzymes. *J. Amer. Chem. Soc.* 93, 4889 (1971).
60. COZZARELLI, N. R., N. E. MELECHEN, T. M. JOVIN, and A. KORNBURG: Polynucleotide cellulose as a substrate for polynucleotide ligase induced by phage T4. *Biochem. Biophys. Res. Comm.* 28, 578 (1967).
61. CRAMER, F.: Probleme der chemischen Polynucleotidsynthese. *Angew. Chem.* 73, 49 (1961).
62. CRAMER, F., H. NEUNHOFFER, K. H. SCHIEIT, G. SCHNEIDER, and J. TENNIGKEIT: Neue Phosphorylierungsreaktionen und Schutzgruppen für Nucleotide. *Angew. Chem.* 74, 387 (1962).
63. CRAMER, F., H. P. BÄR, H. J. RUMSE, W. SAENGER, K. H. SCHIEIT, G. SCHNEIDER, and J. TENNIGKEIT: Stabilität von Schutzgruppen für Nucleoside und Nucleotide. *Tetrahedron Letters* 1039 (1963).
64. CRAMER, F., and K. H. SCHIEIT: Über Benzhydrylester von Nucleotiden. *Liebigs Ann. Chem.* 679, 150 (1964).
65. CRAMER, F.: Die Synthese von Oligo- und Polynucleotiden. *Angew. Chem.* 78, 186 (1966).
66. CRAMER, F., and T. HATA: Chemie der energiereichen Phosphate. XIX. Phosphorylierung mit der Additionsverbindung aus Bromcyanacetamid und Triphenylphosphin. *Liebigs Ann. Chem.* 692, 22 (1966).
67. CRAMER, F., R. HELBIG, H. HETTLER, K. H. SCHIEIT, and H. SELIGER: Oligonucleotidsynthese an einem löslichen Polymeren als Träger. *Angew. Chem.* 12, 640 (1966).
68. CRAMER, F., and H. KÖSTER: Synthesis of oligonucleotides on a polymeric carrier. *Angew. Chem.* 80, 488 (1968). *Angew. Chem. Internat. Edit.* 7, 473 (1968).
69. CRAMER, F.: Chemical synthesis of oligo- and polynucleotides. *Pure Appl. Chem.* 18, 197 (1969).
70. — — — Recent methods of phosphorylation and the application to nucleotide chemistry. *Colloq. Int. Cent. Nat. Rech. Sci.* 343 (1970).
71. CRAMER, F., E. M. GOTTSCHALK, H. MATZURA, K. H. SCHIEIT, and H. STERNBACH: The synthesis of the alternating copolymer poly[r(4'-5'-U)] by RNA polymerase of *Escherichia coli*. *Eur. J. Biochem.* 19, 379 (1971).
72. CUATRECASAS, P.: Protein purification by affinity chromatography. Derivatizations of agarose and polyacrylamide beads. *J. Biol. Chem.* 245, 3059 (1970).
73. CUSACK, N. J., C. B. REESE, and J. H. VAN BOOM: Block synthesis of oligonucleotides by the phosphotriester approach. *Tetrahedron Letters* 2209 (1973).
74. DARLUX, J. L., and P. FROMAGEOT: 5'-Monophosphonucleotides. *Fr. Pat.* 1,539,962 (Cl. C 07D), 20. Sep. 1968, *Appl.* 11. Aug. 1967, *Chem. Abstr.* 72, 321791 (1970).
75. ECKSTEIN, F., and I. RIZK: Oligonucleotidsynthesen mit Phosphorsäure- β , β -trichloräthylester-dichlorid. *Angew. Chem.* 79, 939 (1967).
76. — — — Synthesis of oligonucleotides by use of phosphoric triesters. *Angew. Chem.* 6, 695 (1967).
77. ECKSTEIN, F., and H. GINDL: Polyribonucleotides containing a thiophosphate backbone. *FEBS Letters* 2, 262 (1969).
78. ECKSTEIN, F., and I. RIZK: Synthese von Oligodesoxynucleotiden über Phosphorsäure-triester. *Chem. Ber.* 102, 2362 (1969).
79. ECKSTEIN, F.: Nucleoside phosphorothioates. *J. Amer. Chem. Soc.* 92, 4718 (1970).
80. ECKSTEIN, F., and H. GINDL: Polyribonucleotides containing a phosphorothioate backbone. *Eur. J. Biochem.* 13, 558 (1970).
81. ECKSTEIN, F., and K.-H. SCHIEIT: Procedures in nucleic acid research. 2, 665 (G. L. CANTONI and D. R. DAVIES, eds.). New York: Harper and Row, 1971.
82. ECKSTEIN, F.: Protection of phosphoric and related acids. In: Protective groups in organic chemistry. (J. F. W. McOMIE, ed.). London-New York: Plenum Press, 1973.
83. ENDO, T., K. IKEDA, Y. KAWAMURA, and Y. MIZUNO: I-oxidopyridine-2-yl-diazomethane. A water-soluble alkylating agent for nucleosides and nucleotides. *J. Chem. Soc. D. Chem. Comm.* 673 (1973).
84. ERAMSHKINA, S. A., and A. M. YURKEVICH: 2',3'-O-phenylboric esters of ribonucleosides in the synthesis of diribonucleoside phosphates. *Zh. Obshch. Khim.* 40, 652 (1970).
85. ETO, M., M. SASAKI, M. ITO, M. ETO, and H. OHKAWA: Synthesis of 2-(methylthio)-4H-1,3,2-benzodioxaphosphorine-2-oxide by thiono-thiol conversion and its use as a phosphorylating agent. *Tetrahedron Letters* 4263 (1971).
86. FAERBER, P., K.-H. SCHIEIT, and H. SOMMER: A new poly-nucleotide complex poly-(s^2C). *poly(f)*. *Eur. J. Biochem.* 27, 109 (1972).
87. FALK, W., and C. TAMM: Nucleoside und Nucleotide. Teil 3. Über die Polykondensation von Thymidin-3'-phosphat nach der Triestermethode. *Helv. Chim. Acta* 55, 1928 (1972).
88. FARMER, P. B.: personal communication.
89. FEIX, G., R. POLLET, and C. WEISSMANN: Replication of Viral RNA. XVI. Enzymatic Synthesis of Infectious Viral RNA with Noninfectious Q_{μ} Minus Strands as Template. *Proc. Natl. Acad. Sci. US* 59, 145 (1968).
90. FEIX, G.: Oligoribonucleotides as primer for terminal deoxynucleotidyl transferase. *FEBS Letters* 18, 280 (1971).
91. — — — Enzymatic synthesis of polydeoxynucleotides covalently linked to an oligoribonucleotide primer. *Biochem. Biophys. Res. Commun.* 46, 2141 (1972).
92. — — — Initiation of DNA synthesis by oligoribonucleotides. In: Gene expression and its regulation (F. T. KENNEY, B. A. HANKALO, G. FAVELUKES, and J. T. AUGUST, eds.), p. 301. New York: Plenum Press, 1973.
93. FILIP, J., and L. BOJACEK: Preparation of uridine 5'-monophosphate- s^3H and uridine 5'-monophosphate- 6^3H with high molar activity. *Radioisotopy* 12, 343 (1971).
94. FRANKE, A., F. ECKSTEIN, K. H. SCHIEIT, and F. CRAMER: Synthese von Oligo- und Polynucleotiden. XVI. Synthese von Desoxypolynucleotiden mit der Trichloräthylphosphatgruppe. *Chem. Ber.* 101, 944 (1968).
95. FRANKE, A., K. H. SCHIEIT, and F. ECKSTEIN: Selektive Phosphorylierung von Nucleosiden. *Chem. Ber.* 101, 2998 (1968).

96. FREIST, W., and F. CRAMER: Synthese von Oligonucleotid-5'-phosphaten an einem polymeren Träger mit 2-(α -Pyridyl)-Äthanol als funktioneller Gruppe. *Angew. Chem.* 82, 358 (1970).
97. FREIST, W., and F. CRAMER: Synthese von Oligodesoxynucleotiden mit 2-(α -Pyridyl)-Äthanol als Phosphatschutzgruppe. *Chem. Ber.* 103, 1032 (1970).
98. FREIST, W., R. HEUNG, and F. CRAMER: 2-(α -Pyridyl)-Äthanol als Phosphatschutzgruppe. *Chem. Ber.* 103, 1032 (1970).
99. FROMAGEOT, H. P. M., B. E. GRIFFIN, C. B. REESE, and J. E. SULSTON: Synthesis of oligoribonucleotides. III. Monoacylation of ribonucleosides and derivatives by orthoester exchange. *Tetrahedron* 23, 2315 (1967).
100. FUJIMOTO, Y. (Kyowa Fermentation Industry Co., Ltd.): 2'-3'-O-Substituted ribonucleosides. *Jap. Pat.* 68 25,496 (Cl. 16 E 362), 4. Nov. 1968, *Appl.* 10. Nov. 1965. *Chem. Abstr.* 70, 68707 m (1969).
101. —: 2'-3'-O-Substituted ribonucleosides. *Jap. Pat.* 68 25,498 (Cl. 16 E 431), 4. Nov. 1968, *Appl.* 10. Nov. 1965. *Chem. Abstr.* 70, 68708 n (1969).
102. GALLO, R. C.: Reverse Transcriptase — The DNA Polymerase of Oncogenic RNA Viruses. *Nature* 234, 194 (1973).
103. GASSEN, H. G.: Synthesis by ribonuclease A of codons containing modified nucleosides in the "wobble" position. *FEDS Letters* 14, 225 (1971).
104. GASSEN, H. G., and R. NOLTE: Synthesis by polymer-bound ribonuclease of the termination codons U-A-A, U-A-G, and U-G-A. *Biochem. Biophys. Res. Commun.* 44, 1410 (1971).
105. GASSEN, H. G.: personal communication.
106. GILLAM, S., and M. SMITH: Enzymatic synthesis of deoxyribo-oligonucleotides of defined sequence. *Nature* 238, 233 (1972).
107. GLASER, R., U. SEQUIN, and C. TAMM: Nucleoside und Nucleotide. Festphasensynthese von Oligonucleotiden an einem unlöslichen, makroporösen Träger. *Helv. Chim. Acta* 56, 654 (1973).
108. GLINSKI, R. P., A. B. ASH, C. L. STEVENS, M. B. SPORN, and H. M. LAZARUS: Nucleotide synthesis. I. Derivatives of thymidine containing p-nitrophenyl phosphate groups. *J. Org. Chem.* 36, 245 (1971).
109. GLINSKI, R. P., C. C. BACON, and C. L. STEVENS: Synthesis of partially protected oligonucleotides. Presented at 161st National Meeting of American Chemical Society, Los Angeles, Calif., No. CARB 29 (1971).
110. GOLDBERG, I. H.: Preparation and properties of polypseudouridylic acid. In: *Methods in enzymology* (L. GROSSMAN and K. MOLDAVE, eds.), XII B, 519. New York-London: Academic Press, 1968.
111. GOULIAN, M., A. KORNBERG, and R. L. SINSHEIMER: Enzymatic Synthesis of DNA. XXIV. Synthesis of Infectious Phage ϕ X 174 - DNA. *Proc. Natl. Acad. Sci.* 58, 2321 (1967).
112. GRAMS, G. W., and R. L. LETSINGER: N⁶,3'-O-disubstituted deoxyadenosine. *J. Org. Chem.* 33, 2589 (1968).
113. —: Synthesis of a diribonucleoside monophosphate by the β -cyanoethyl phosphotriester method. *J. Org. Chem.* 35, 868 (1970).
114. GRANT, R. C., S. J. HARWOOD, and R. D. WELLS: The synthesis and characterization of poly d(I-C) - poly d(I-C). *J. Amer. Chem. Soc.* 90, 4474 (1968).
115. GRANT, R. C., M. KODAMA, and R. D. WELLS: Enzymatic and physical studies on (dI-dC)_n (dI-dC)_n and (dG-dC)_n. *Biochemistry* 11, 805 (1972).
116. GREEN, B. P. L., T. RAVINDRANATHAN, C. B. REESE, and R. SAFFILL: Synthesis of oligoribonucleotides. VIII. Preparation of ribonucleoside 2',5'-bisacetals. *Tetrahedron Letters* 1031 (1970).

117. GRIFFIN, B. E., M. JARAMAN, C. B. REESE, and R. E. SULSTON: The synthesis of oligoribonucleotides. II. Methoxymethylidene derivatives of ribonucleosides and 5'-ribonucleotides. *Tetrahedron* 23, 2301 (1967).
118. GRINEVA, N. I., V. F. ZARYTOVA, D. G. KNORRE, and E. V. YARMOLINSKAYA: Alkylating derivatives of nucleic acid components. XI. Mechanism of formation of benzylidene derivatives of nucleotides. *Izv. Sib. Otd. Akad. Nauk. Chem. Abstr.* 76, 107 (1971).
119. GRÜNBARGER, D., A. HOLY, and F. ŠORAI: Synthesis of Tribrbonucleoside Diphosphates with Ribonuclease T₁. *Coll. Czech. Chem. Comm.* 33, 286 (1968).
120. GUPTA, N. K., E. OHTSUKA, H. WEBER, S. H. CHANG, and H. G. KUDRANA: Studies on Polynucleotides. LXXXVII. The Joining of Short Deoxyribopolynucleotides by DNA Joining Enzymes. *Proc. Natl. Acad. Sci. US* 60, 285 (1968).
121. GUPTA, N. K., E. OHTSUKA, V. SGARANIELLA, H. BÜCHT, A. KUMAR, H. WEBER, and H. G. KUDRANA: Studies on Polynucleotides. LXXXVIII. Enzymatic Joining of Chemically Synthesized Segments Corresponding to the Gene for Alanine Transfer RNA. *Proc. Natl. Acad. Sci. US* 60, 1338 (1968).
122. HAEFFNER, E. W.: Studies on the thermic phosphorylation of activated nucleoside by phosphate anion and nucleotide anion. *Biochim. Biophys. Acta* 212, 182 (1970).
123. HACHIMANN, J., and H. G. KUDRANA: Studies on polynucleotides. XCIII. A further study of the synthesis of deoxyribopolynucleotides using preformed oligonucleotide blocks. *J. Amer. Chem. Soc.* 91, 2749 (1969).
124. HAGA, K., M. KAINOSHO, and M. YOSHIKAWA: Phosphorylation. V. Synthesis of inosine-5'-thiophosphates. *Bull. Chem. Soc. Jap.* 44, 460 (1973).
125. HAGENER, L., H. G. GASSEN, and H. MATTHIAS: Synthesis and coding properties of poly(c'A), poly(c'A), poly(c'A) and poly(h'A). *Biochem. Biophys. Res. Commun.* 50, 1104 (1973).
126. HALMANN, M., R. A. SANCHEZ, and L. E. ORGEL: Phosphorylation of D-ribose in aqueous solution. *J. Org. Chemistry* 34, 3702 (1969).
127. HANSBURY, E., V. N. KERR, V. E. MITCHELL, R. L. RATLIFF, D. A. SMITH, D. L. WILLIAMS, and F. N. HAYES: Synthesis of polydeoxynucleotides using chemically modified subunits. *Biochim. Biophys. Acta* 199, 322 (1970).
128. HARVEY, C. L., E. M. CLERICUZIO, and A. L. NUSSBAUM: Small-scale preparation of 5'-nucleotides and analogs by carboxyphosphotransferase. *Anal. Biochem.* 36, 413 (1970).
129. HARVEY, C. L., R. WRIGHT, A. F. COOK, D. T. MAICHUK, and A. L. NUSSBAUM: Use of phosphate-blocking groups in ligation of oligodeoxynucleotides. *Biochemistry* 12, 208 (1973).
130. HARVEY, C. L., R. WRIGHT, and A. L. NUSSBAUM: Lambda phage DNA: Joining of a chemically synthesized cohesive end. *Science* 179, 291 (1973).
131. HARVEY, C. L., A. DE CZEKALA, A. F. COOK, M. J. HOLMAN, T. F. GABRIEL, J. E. MICHIALEWSKY, and A. L. NUSSBAUM: High pressure liquid chromatography applied to gene synthesis. *Biochim. Biophys. Acta* 324, 433 (1973).
132. HASHIZUME, T.: Synthesis of biochemically significant organic phosphate compounds. I. Bis-p-nitrophenyl-phosphorochloridate as a phosphorylating agent. *Mem. Coll. Agr., Kyoto Univ., Chem. Ser.* 81, 1 (1959). *Chem. Abstr.* 57, 14157 (1962).
133. HATA, T., and J. AZIZIAN: 2-chloroethyl orthoformate as a reagent for protection in nucleotides synthesis. *Tetrahedron Letters* 4443 (1969).
134. HATA, T., Y. MUSHIKA, and T. MUKAIYAMA: New phosphorylating reagent. I. Preparation of alkyl dihydrogen phosphates by means of 2-chloromethyl-4-nitrophenyl phosphorodichloridate. *J. Amer. Chem. Soc.* 91, 4532 (1969).
135. —: —: New phosphorylating reagent. II. Preparation of mixed diesters of phosphoric acid by the use of alkyl-2-chloromethyl-4-nitrophenyl hydrogen phosphate. *Tetrahedron Letters* 3505 (1970).

136. HATA, T., K. TAJIMA, and T. MUKAIYAMA: Simple protecting group protection-purification "handle" for polynucleotide synthesis. I. *J. Amer. Chem. Soc.* 93, 4928 (1971).
137. HATA, T., and K. J. CHONG: p-Nitrophenyl phosphate as a phosphorylating reagent in nucleotide synthesis. *Bull. Chem. Soc. Jap.* 45, 654 (1972).
138. HATA, T., I. NAKAGAWA, and N. TAKEBAYASHI: Simple protecting group protection-purification handle for polynucleotide synthesis. III. New method for the synthesis of dinucleotides. *Tetrahedron Letters* 2931 (1972).
139. HAYASHI, H., and F. EGAMI: Fractionation and Properties of Guanidyl acid Polymers synthesized by Ribonuclease T₁. *J. Biochem. (Tokyo)* 53, 176 (1963).
140. HAYATSU, H., and H. G. KIKORANA: Deoxyribooligonucleotide synthesis on a polymer support. *J. Amer. Chem. Soc.* 88, 3182 (1966).
141. — — — Studies on Polynucleotides. LXXII. Deoxyribooligonucleotide synthesis on a polymer support. *J. Amer. Chem. Soc.* 89, 3880 (1967).
142. HAYES, F. N., V. E. MITCHELL, R. L. RATLIFF, and D. L. WILLIAMS: Limited enzymatic addition of deoxyribonucleotide units onto chemically synthesized oligodeoxyribonucleotides. *Biochemistry* 6, 2488 (1967).
143. HAYES, F. N., E. HANSBURY, V. E. MITCHELL, R. L. RATLIFF, and D. L. WILLIAMS: Synthesis of N-acetylated deoxyribonucleoside 5'-triphosphates and their utilization in enzymatic formation of single-stranded polydeoxyribonucleotides. *Eur. J. Biochem.* 6, 485 (1968).
144. HAYES, F. N., and V. E. MITCHELL: Gel filtration chromatography of polydeoxynucleotides using agarose columns. *J. Chromatog.* 39, 139 (1969).
145. HEIMER, E. P., M. AHMAD, and A. L. NUSSBAUM: Chemical synthesis of the "sticky end" of lambda phage DNA r-strand. *Biochem. Biophys. Res. Commun.* 48, 348 (1972).
146. HEIMER, E., M. AHMAD, S. ROY, A. RAMEL, and A. L. NUSSBAUM: Nucleoside S-alkyl phosphorotriates. VI. Synthesis of deoxyribonucleotide oligomers. *J. Amer. Chem. Soc.* 94, 1707 (1972).
147. HELRIC, R.: Oligonucleotidsynthesen am polymeren Träger. Dissertation. Techn. Hochschule Braunschweig. 1967.
148. HOBBS, J., H. STERNBACH, and F. ECKSTEIN: Poly 2'-deoxy-2'-chlorouridylic and -cytidylic acids. *FEBS Letters* 15, 345 (1971).
149. — — — Poly 2'-deoxy-2'-aminouridylic acid. *Biochem. Biophys. Res. Commun.* 46, 1509 (1972).
150. HOBBS, J., H. STERNBACH, M. SPRINZL, and F. ECKSTEIN: Polynucleotides containing 2'-chloro-2'-deoxyribose. *Biochemistry* 11, 4336 (1972).
151. — — — Polynucleotides containing 2'-amino-2'-deoxyribose and 2'-azido-2'-deoxyribose. *Biochemistry* 12, 5138 (1973).
152. HOLY, A., and J. SMRT: Oligonucleotide compounds. XV. A general approach to the stepwise synthesis of ribooligonucleotides. Synthesis of some triribonucleoside diphosphates. *Coll. Czech. Chem. Commun.* 31, 3800 (1966).
153. HOLY, A., S. CHLADEK, and J. ZEMLIČKA: Oligonucleotide compounds. XXIX. Reactions of ribonucleoside 2'(3')-phosphates with dimethylformamide acetals. *Collect. Czech. Chem. Commun.* 34, 253 (1969).
154. HOLY, A., and J. ZEMLIČKA: Oligonucleotide compounds. XXXIII. A study on hydrolysis of N-dimethylaminomethylene-cytidine, -adenosine, -guanosine and related 2'-deoxy compounds. *Collect. Czech. Chem. Commun.* 34, 2449 (1969).
155. — — — Oligonucleotide compounds. XXXV. Reaction of diribonucleoside phosphates with dimethylformamide acetals. *Collect. Czech. Chem. Commun.* 34, 3921 (1969).
156. HOLY, A., and G. KOWOLLIK: Nucleic acid components and their analogs. CXXXI. Simple enzymic synthesis of nucleoside-5'-phosphates. *Coll. Czech. Chem. Commun.* 35, 1013 (1970).
157. HOLY, A., and M. SOUCEK: Benzoyl cyanide — new benzoylating agent in nucleoside and nucleotide chemistry. *Tetrahedron Letters* 185 (1971).
158. HOLY, A.: Phosphorylation of nucleosides with trichloromethylphosphonic acid derivatives. *Tetrahedron Letters* 157 (1972).
159. HONJO, M., Y. FURUKAWA, K. KOBAYASHI, and R. MARUNOTO (Takeda Chem. Industries, Ltd.): N-Acyl-2',3',5'-tri-O-acyl-cytidines. *Ger. Offen.* 2,038,807 (Cl. C 07d). 18. Feb. 1971. *Appl.* 6. Aug. 1969. *Chem. Abstr.* 74, 100354q (1971). *Chem. Abstr.* 74, 100354d (1971).
160. HONJO, M., S. YOSHIKAWA, K. KOBAYASHI, and R. MARUNOTO: N⁴,2',3',5'-tetra-alkanoyletylides. *Japan Patent* 71 37,827 (1971). *Chem. Abstr.* 76, 34533k (1972).
161. HUDSON, R. F., and M. GREEN: Die Stereochemie von Substitutionsreaktionen am Phosphor. *Angew. Chem.* 75, 47 (1963).
162. ICHIHARA, M., and K. MURAO: Nucleosides and Nucleotides. XXXVIII. Synthesis of 8-oxoguanosine nucleotides and uric acid-9-D-ribose-5'-phosphate. *Chem. Pharm. Bull. (Tokyo)* 16, 1330 (1968).
- 162a. ICHIHARA, M., and S. UESUGI: Selective Tosylation of Adenosine-5'-phosphate. *Tetrahedron Letters* 713 (1970).
- 162b. — — — Studies on Nucleosides and Nucleotides. LIII. Purine Cyclonucleosides. XVIII. Selective Tosylation of Adenine Nucleotides. Synthesis of 8,2'-Anhydro-8-mercapto-9-β-D-arabinofuranosyl adenine and 5'- and 3'-5'-cyclic phosphate. *Tetrahedron* 28, 3687 (1972).
- 162c. — — — Studies on Nucleosides and Nucleotides. LV. Reaction of Cytidine-5'-monophosphate with p-Toluene-sulfonylchloride. *Chem. Pharm. Bull. (Tokyo)* 21, 264 (1973).
163. INURA, N., T. TSURUO, and T. UKITA: On the benzylation of nucleosides. I. Reaction of uridine with benzyl bromide in the presence of sodium hydride. *Chem. Pharm. Bull. (Tokyo)* 16, 1105 (1968).
164. IRIE, S.: Selective phosphorylation of thionucleosides. *J. Biochem. (Tokyo)* 68, 129 (1970).
165. IRIE, S., T. UCHIDA, and F. EGAMI: Synthesis and ribonuclease degradation of dinucleoside monophosphates containing a thionucleoside. *Biochim. Biophys. Acta* 289, 289 (1970).
166. JACOB, T. M., and H. G. KHORANA: Studies on polynucleotides. XXX. A comparative study of reagents for the synthesis of the C₃-C₂ internucleotide linkage. *J. Amer. Chem. Soc.* 86, 1630 (1964).
167. JANIK, B., M. P. KOTICK, T. H. KREISER, L. F. REVERMAN, R. G. SOMMER, and D. P. WILSON: Synthesis and properties of poly 2'-fluoro-2'-deoxyuridylic acid. *Biochem. Biophys. Res. Commun.* 46, 1153 (1972).
168. JANIK, B., R. G. SOMMER, M. P. KOTICK, D. P. WILSON, and R. K. ERICKSON: Synthesis and properties of poly(1,N⁶-ethenoadenylic acid) and poly(3,N²-ethenocytidylic acid). *Physiol. Chem. Phys.* 5, 27 (1973).
169. JANION, C., and D. SIUGAR: Mechanism of hydroxylamine mutagenesis: Complexing properties of copolymers of hydroxycytidylic acid with cytidylic or uridylic acids. *Acta Biochim. Polon.* 16, 219 (1969).
170. JOVIN, T. M., and A. KORNBERG: Oligonucleotide celluloses as solid state primers and templates for polymerases. *J. Biol. Chem.* 243, 250 (1968).
171. KABACHNIK, M. M., I. A. POLYAKOVA, V. K. POTAPOV, Z. A. SIABAROVA, and M. A. PROKOF'EV: Phosphorylation of nucleosides on polymeric carriers. *Dokl. Akad. Nauk SSSR* 195, 1344 (1970).
172. KABACHNIK, M. M., V. K. POTAPOV, Z. A. SIABAROVA, and M. A. PROKOF'EV: Oxidative phosphorylation of nucleosides. *Dokl. Akad. Nauk SSSR* 195, 1107 (1970).
173. — — — A new method of synthesis of internucleotide bonds using polymeric supports. *Dokl. Akad. Nauk SSSR* 201, 858 (1971).

174. KAPACHNIK, M. M., N. G. TMOEEVA, M. V. BUDANOV, V. K. POTAPOV, Z. A. SHABAROVA, and M. A. PROKOF'EV: Synthesis of oligonucleotides on a polymer carrier. *Zhur. Obshch. Khim.* 43, 379 (1973).
175. KAPULER, A. M., C. MONNY, and A. M. MICHELSON: The relationship of mono- and polynucleotide conformation to catalysis by polynucleotide phosphorylase. *Biochim. Biophys. Acta* 217, 18 (1970).
176. KATAGIRI, N., C. P. BAHU, K. ITRAKURA, J. MICHIEWICZ, and S. A. NARANG: Use of 9-fluorenylmethanol as phosphate protecting group in the synthesis of deoxyribonucleotides. *J. Chem. Soc. D, Chem. Comm.* 803 (1973).
- 176a. KATAGIRI, N., K. ITRAKURA, and S. A. NARANG: Novel condensing reagents for polynucleotide synthesis. *J. Chem. Soc. D, Chem. Comm.* 325 (1974).
177. KATHAWALA, F., and F. CRAMER: Synthesis von Oligo- und Polynucleotiden. XIII. 2',3'-(2,4-Dimethoxybenzyliden-) als Phosphatschutzgruppe. *Liebigs Ann. Chem.* 709, 185 (1967).
178. — — — Synthese von Oligo- und Polynucleotiden. XIV. Darstellung von Desoxyoligonucleotiden mit 2',3'-(2,4-Dimethoxybenzyliden-)uridin als Phosphatschutzgruppe. *Liebigs Ann. Chem.* 712, 195 (1968).
- 178a. KAUFMANN, G., and U. Z. LITTAUER: Deoxyadenosine diphosphate as substrate for polynucleotide phosphorylase from *Escherichia coli*. *FEBS Letters* 2, 79 (1969).
179. KAUFMANN, G., M. FRIDKIN, A. ZUTRA, and U. Z. LITTAUER: Monofunctional substrates of polynucleotide phosphorylase. *Eur. J. Biochem.* 24, 4 (1971).
180. KAUFMANN, G., A. ZUTRA, and U. Z. LITTAUER: Synthesis of the heptanucleotide U-U-U-G-A-A-G using isovaleryl nucleoside diphosphates and sepharose bound polynucleotide phosphorylase. *Israel J. Chem.* 9, 44 BC (1971).
181. KAVUNENKO, A. P., E. N. MOROZOVA, and N. S. TIKHOMIROVA-SIDOROVA: Preparation of purine-pyrimidine dinucleotides with terminal 2',3'-cyclophosphate and their use for the synthesis of trinucleoside diphosphates. *Zh. Obshch. Khim.* 41, 226 (1971).
182. KAVUNENKO, A. P., V. P. SUKHAREVICH, and N. S. TIKHOMIROVA-SIDOROVA: Water-soluble carbodiimide in oligoribonucleotide synthesis catalyzed by pancreatic ribonuclease. *Zh. Obshch. Khim.* 41, 679 (1971).
183. KELLY, R. C., W. J. WECHESTER, and D. T. GIST (Upjohn Co.): 5'-O-Derivatives of aracytidine. *Ger. Offen.* 2,025,624 (Cl. C 07d), 3. Dec. 1970, US Appl. 27. May 1969 — 16. Feb. 1970. *Chem. Abstr.* 74, 54151 w (1971).
184. KHORANA, H. G.: Recent developments in the chemistry of phosphate esters of biological interest. New York: John Wiley & Sons, Inc. 1961.
185. — — — Synthesis in the study of nucleic acids. *Proc. 7th Intern. Congr. Biochem.*, p. 17, Tokyo (1967).
186. — — — Polynucleotide synthesis and the genetic code. *The Harvey Lectures* 62, 79 (1968).
187. — — — Nucleinsäuresynthese als Werkzeug für das Studium des Genetischen Codes (Nobel-Vortrag). *Angew. Chem.* 81, 1027 (1969).
188. — — — Total synthesis of the gene for an alanine transfer ribonucleic acid from yeast. *Pure Appl. Chem.* 25, 91 (1971).
189. KHORANA, H. G., K. L. AGARWAL, H. BÜCHI, M. H. CARUTHERS, N. K. GUPTA, K. KLEPPE, A. KUNAR, E. OHTSUKA, U. L. RAJ BHANDARY, J. H. VAN DE SANDE, V. SGARAMELLA, T. TERAO, H. WEBER, and T. YAMADA: Studies on polynucleotides. CIII. Total synthesis of the structural gene for an alanine transfer ribonucleic acid from yeast. *J. Mol. Biol.* 72, 209 (1972).
190. KHORANA, H. G., K. L. AGARWAL, P. BESMER, H. BÜCHI, M. H. CARUTHERS, P. J. CASHION, M. FRIDKIN, E. JAY, D. G. KLEID, A. KUNAR, P. C. LOEWEN, R. MILLER, K. MINAMOTO, R. RAMA MOORTHY, A. PANET, J. H. VAN DE SANDE, T. SEKIYA, and N. SIDOROVA: Synthesis of the gene for the precursor of *E. coli* tyrosine suppressor tRNA. Abstracts of the 166th Meeting of the Amer. Chem. Soc. (1973).
191. KIWAWA, T. A., and C. B. REESE: Phosphorylation of nucleosides with *o*-phenylene-phosphorochloridate and *o*-phenylene phosphate. *Tetrahedron* 27, 6189 (1971).
192. KIKUGAWA, K., F. SATO, T. TSURUO, N. IMURA, and T. UKITA: On the benzoylation of nucleosides. II. A novel synthesis of 2'-*O*-benzyl uridine. *Chem. Pharm. Bull. (Tokyo)* 16, 1110 (1968).
193. KIMHI, Y., and U. Z. LITTAUER: Polynucleotide phosphorylase from *Escherichia coli*. In: *Methods in enzymology* XII B, 513 (L. GRUNSMAN and K. MOLDAVE, eds.). New York-London: Academic Press, 1968.
194. KLEE, C. B.: Procedures in nucleic acid research. 2, 896 (G. L. CANTONI and D. R. DAVIES, eds.). New York: Harper and Row, 1971.
195. KLEFFE, K., J. H. VAN DE SANDE, and H. G. KHORANA: Polynucleotide ligase-catalyzed joining of deoxyribo-oligonucleotides on ribopolynucleotide templates and of ribonucleotides on deoxyribopolynucleotide templates. *Proc. Nat. Acad. Sci.* 67, 68 (1970).
196. KNORR, D. G., E. F. MISHENINA, T. I. SHUMINA: Copolymer of acrylamide and 5'-*C*-acrylylguanosine-2',3'-cyclophosphate as a substrate of guanyl-RNase during formation of the internucleotide bond. *Dokl. Akad. Nauk. SSSR* 198, 1089 (1970).
197. KOCIETKOV, N. K., and E. J. BUDOVSKI: Organic chemistry of nucleic acids. London-New York: Plenum Press, 1971.
198. KOGAN, E. M., E. N. MOROZOVA, N. S. TIKHOMIROVA-SIDOROVA, and G. E. USTYUZHANIN: Synthesis of purine-pyrimidine triphosphonucleotides in the presence of pancreatic ribonuclease. *Zh. Obshch. Khim.* 39, 2576 (1969).
199. KOIKE, T., T. UCHIDA, and F. EGAMI: Synthesis of guanylyl-(3',5')-nucleosides and oligoguanilyc acids by ribonuclease N₁. *Biochim. Biophys. Acta* 190, 257 (1969).
200. KOIKE, T., T. UCHIDA, and F. EGAMI: Synthesis of adenylyl-(3',5')-nucleosides, adenylyl-(3',5')-guanosine 2',3'-cyclic phosphate, and oligoadenylic acids by ribonuclease U₂. *J. Biochem. (Tokyo)* 69, 111 (1971).
201. KOIKE, T., T. UCHIDA, and F. EGAMI: Synthesis of oligo-ApGp and other oligonucleotides by ribonuclease N₁. *J. Biochem. (Tokyo)* 70, 55 (1971).
202. KOLODKINA, I. I., A. S. GUSEVA, E. A. IVANOVA, L. S. VARSHAVSKAYA, and A. M. YURKEVICH: Synthesis and properties of areneboronates of nucleosides and nucleotides. *Zh. Obshch. Khim.* 40, 2489 (1970).
203. KOLODKINA, E. E., E. A. IVANOVA, and A. M. YURKEVICH: Ion-exchange chromatography of arylboronic acid complexes of nucleosides and mononucleotides. *Khim. Priir. Soedin.* 6, 612 (1970). *Chem. Abstr.* 74, 112363 e (1971).
204. KORNBERG, A.: Adenosine phosphokinase. In: *Methods in Enzymology*, Vol. II. (S. P. COLOWICK and N. O. KAPLAN, eds.), p. 497. New York: Academic Press, 1955.
205. KÖSEL, H., H. BÜCHI, and H. G. KHORANA: Studies on polynucleotides. LXV. Total synthesis of deoxyribopolynucleotides containing repeating tetranucleotide sequences. *J. Amer. Chem. Soc.* 89, 2185 (1967).
206. KÖSEL, H., A. R. MORGAN, and H. G. KHORANA: Studies on polynucleotides. LXXIII. Synthesis in vitro of polypeptides containing repeating tetrapeptide sequences dependent upon DNA-like polymers containing repeating tetranucleotide sequences: direction of reading of messenger RNA. *J. Mol. Biol.* 26, 449 (1967).
207. KÖSEL, H., M. W. MOON, and H. G. KHORANA: Studies on Polynucleotides LX. The Use of Preformed Dinucleotide Blocks in the Stepwise Synthesis of Deoxyribopolynucleotides. *J. Amer. Chem. Soc.* 89, 2148 (1967).
208. KÖSEL, H., and R. ROYCHOUPTURY: Synthetic polynucleotides. The terminal addition of riboadenylic acid to deoxyoligonucleotides by terminal deoxynucleotidyl transferase as a tool for the specific labelling of deoxyoligonucleotides at the 3'-ends. *Eur. J. Biochem.* 22, 271 (1971).

- 208a. KÖSEL, H., and R. ROYCHODURKY: Proofreading function of DNA polymerase I from *E. coli*. Nature of excision of ribonucleotides from the 3'-termini of oligodeoxynucleotide primers. *J. Biol. Chem.* 249, 4094 (1974).
209. KÖSEL, H., and S. KÜHN: unpublished.
210. KÖSTER, H.: Polymer support oligonucleotide synthesis. VI. Inorganic carriers. *Tetrahedron Letters* 1527 (1972).
211. KÖSTER, H., and K. HEYNS: Polymer support oligonucleotide synthesis. VII. Use of Sephadex LH 20. *Tetrahedron Letters* 1531 (1972).
212. KÖSTER, H.: Polymer support oligonucleotide synthesis. VIII. Use of polyethylene glycol. *Tetrahedron Letters* 1535 (1972).
213. KÖSTER, H., and F. CRAMER: Synthese von Oligonucleotiden an einem Polycorn-Polystyrol als polymerem Träger. *Liebigs Ann. Chem.* 766, 6 (1972).
214. KÖSTER, H., and S. GEUSSENHÄNER: Ein neuer Träger für die Festphasensynthese von Oligomeren. *Angew. Chem.* 84, 712 (1972).
215. KÖSTER, H., and F. CRAMER: Reaktionskinetische Untersuchungen an einem makroporösen unquellbaren Polystyrol: Abspaltung von Nucleosiden und Nucleotiden, die über p-Anisylidiphenylmethyletherbindung an das Polymerisat gebunden sind. *Makromol. Chem.* 167, 171 (1973).
216. KÖSTER, H., and W. HEIDMANN: A new approach to the synthesis of oligodeoxynucleotides. *Angew. Chem.* 85, 871 (1973). *Angew. Chem. Internat. Edit.* 12, 859 (1973).
217. KÖSTER, H., and F. CRAMER: Synthese von Desoxyoligonucleotiden an einem makroporösen Polystyrol. *Liebigs Ann. Chem.* 946 (1974).
218. KÖSTER, H., F. POLLACK, and F. CRAMER: Synthese der Desoxyoligonucleotide d(TpdT) und d(TpTdApdCpdTpA) an einem makroporösen Polystyrol. *Liebigs Ann. Chem.* 959 (1974).
- 218a. KOWOLLIK, G., K. GAERTNER, and P. LANGEN: 2'- and 3'-O-trityluridine. *Tetrahedron Letters* 3345 (1972).
219. KUMAR, A., E. OHTSUKA, and H. G. KHORANA: Studies on Polynucleotides. CVI. Total synthesis of the structural gene for an alanine transfer ribonucleic acid from yeast. Synthesis of two nonanucleotides and a heptanucleotide corresponding to nucleotide sequences 22 to 30, 41 to 49 and 28 to 34. *J. Mol. Biol.* 72, 289 (1972).
- 219a. KUMAR, A., and H. G. KHORANA: Studies on Polynucleotides. CVIII. Total synthesis of the structural gene for an alanine transfer ribonucleic acid from yeast. Synthesis of an undecadeoxynucleotide, a decadeoxynucleotide and an octadeoxynucleotide corresponding to the nucleotide sequences 7 to 27. *J. Mol. Biol.* 72, 329 (1972).
220. KUSAMA, T., and H. HAYATSU: Use of a derivatized Merrifield resin for the polymer-supported synthesis of oligodeoxynucleotides. *Chem. Pharm. Bull. (Tokyo)* 18, 319 (1970).
221. LAPIDOT, Y.: Chromatography of dinucleoside monophosphates on a Bio-Gel P-4 column. *J. Chromatog.* 56, 143 (1971).
222. LAPIDOT, Y., and I. BARZILAY: The separation of 2'-5' dinucleoside monophosphates from the corresponding 3'-5' isomers on a DEAE-sephadex A-25 column. *J. Chromatog.* 71, 275 (1972).
223. LAPIDOT, Y., I. BARZILAY, and D. SALOMON: Ion-exchange thin-layer chromatography and paper ionophoresis of dinucleoside monophosphates. *Anal. Biochem.* 49, 301 (1972).
224. LEHRACH, H., and K. H. SCHIEB: Synthesis and properties of a new fluorescent polynucleotide, poly(1,N⁶-ethenoadenylic acid). *Biochim. Biophys. Acta* 308, 28 (1973).
225. LEHRFELD, J.: Silicagel catalyzed detritylation of some carbohydrate derivatives. *J. Org. Chemistry* 32, 2544 (1967).
226. LETSINGER, R. L., M. J. KORNET, V. MAHADEVAN, and D. M. JERINA: Reactions on polymer supports. *J. Amer. Chem. Soc.* 86, 5163 (1964).

227. LETSINGER, R. L., and V. MAHADEVAN: Oligonucleotide synthesis on a polymer support. *J. Amer. Chem. Soc.* 87, 3526 (1965).
228. LETSINGER, R. L., and V. MAHADEVAN: Stepwise synthesis of oligodeoxynucleotides on an insoluble polymer support. *J. Amer. Chem. Soc.* 88, 5319 (1966).
229. LETSINGER, R. L., and K. K. OGILVIE: Use of p-nitrophenyl chloroformate in blocking hydroxyl groups in nucleosides. *J. Org. Chem.* 32, 296 (1966).
- 229a. LETSINGER, R. L., M. H. CARUTHERS, and D. M. JERINA: Reactions of nucleosides on polymer supports. Synthesis of thymidyl-thymidyl-thymidine. *Biochemistry* 6, 1379 (1967).
230. LETSINGER, R. L., M. H. CARUTHERS, P. S. MILLER, and K. K. OGILVIE: Oligonucleotide syntheses utilizing β -benzoylpropionyl, a blocking group with a trigger for selective cleavage. *J. Amer. Chem. Soc.* 89, 7146 (1967).
231. LETSINGER, R. L., and D. M. JERINA: Reactivity of ester groups on insoluble polymer supports. *J. Polymer Science* 5, 1977 (1967).
232. LETSINGER, R. L., and K. K. OGILVIE: A convenient method for stepwise synthesis of oligothymidylate derivatives in large-scale quantities. *J. Amer. Chem. Soc.* 89, 4801 (1967).
233. LETSINGER, R. L., P. S. MILLER, and G. W. GRAVES: Selective N-debenzoylation of N,O-polybenzoylnucleosides. *Tetrahedron Letters* 2621 (1968).
234. LETSINGER, R. L., and P. S. MILLER: Protecting groups for nucleosides used in synthesizing oligonucleotides. *J. Amer. Chem. Soc.* 91, 3356 (1969).
235. LETSINGER, R. L., and K. K. OGILVIE: Synthesis of oligothymidylates via phosphotriester intermediates. *J. Amer. Chem. Soc.* 91, 3350 (1969).
236. LETSINGER, R. L., K. K. OGILVIE, and P. S. MILLER: Developments in syntheses of oligodeoxynucleotides and their organic derivatives. *J. Amer. Chem. Soc.* 91, 3360 (1969).
237. LETSINGER, R., and W. S. MUNGALL: Phosphoramidate analogs of oligonucleotides. *J. Org. Chem.* 35, 3800 (1970).
238. LETSINGER, R. L., and H. H. SELIGER: Polymers with hydroxyl groups as supports for oligonucleotide synthesis. *Macromol. Preprints. XXIIIrd Internat. Congr. of pure and applied chemistry*, Boston, 1261 (1971).
239. LEVINA, A. S., V. K. POTAPOV, D. G. KNORRE, Z. A. SHABAROVA, and T. M. SHUMINA: Individual stages of oligonucleotide synthesis on a highly crosslinked polymer support. *Izv. Sib. Otd. Akad. Nauk.* 117 (1972).
240. LEZIUS, A. G., and K. H. SCHIEB: Enzymatic synthesis of DNA with 4-thiothymidine triphosphate as substitute for dTTP. *Eur. J. Biochem.* 3, 85 (1967).
241. LEZIUS, A. G.: Synthesis and characterization of a copolymer consisting of alternating deoxyadenosine- and 2-thiothymidine nucleotides. *Eur. J. Biochem.* 14, 154 (1970).
242. LEZIUS, A. G., and E. M. GOTTSCHALK: Über eine reversible kooperative Konformationsumwandlung einer synthetischen DNA unter dem Einfluß hoher Salzkonzentrationen. *Hoppe Seyler's Zeitschr. Physiol. Chem.* 351, 413 (1970).
243. LEZIUS, A. G., and U. RATH: Synthesis of Poly[d(A-S⁴T).d(A-S⁴T)] by *Bacillus Subtilis* DNA Polymerase. *Eur. J. Biochem.* 24, 163 (1971).
244. LEZIUS, A. G., and E. DOMIN: A Wobbly Double-Helix. *Nature, New Biol.* 254, 169 (1973).
245. LICHTENTHALER, F. W.: The chemistry and properties of enol phosphates. *Chem. Rev.* 61, 607 (1963).
246. LLOYD, G. S., C.-M. HSU, and B. S. COOPERMAN: On the reactivity of phosphorylimidazole, an analog of known phosphorylated enzymes. *J. Amer. Chem. Soc.* 93, 4889 (1971).
247. LOEWEN, P. C., and H. G. KHORANA: Studies on Polynucleotides. CXXII. The Dodecanucleotide Sequence Adjoining the CCA-End of the Tyrosine Transfer Ribonucleic Acid Gene. *J. Biol. Chem.* 248, 3489 (1973).

248. LOHRMANN, R., and H. G. KIORANA: Studies on Polynucleotides. LII. The use of 2,4,6-trisopropylbenzenesulfonyl chloride for the synthesis of internucleotide bonds. *J. Amer. Chem. Soc.* 88, 829 (1966).
249. LOHRMANN, R., D. SÖLL, H. HAYATSU, E. OHTSUKA, and H. G. KIORANA: Studies on polynucleotides. LI. Syntheses of the 64 possible ribonucleotides derived from the four major ribomononucleotides. *J. Amer. Chem. Soc.* 88, 819 (1966).
250. LOHRMANN, R., and L. E. ORCEV: Urea — inorganic phosphate mixtures as prebiotic phosphorylating agents. *Science* 171, 490 (1971).
251. LUK, D. C. M., P. BARTL, and A. L. NUSSBAUM: Chain length characterization of oligodeoxyribonucleotides by analytical ultracentrifugation. *Anal. Biochem.* 52, 118 (1973).
252. MACKAY, J. K., and P. T. GLUHAN: New approach to the synthesis of polyribonucleotides of defined sequence. *Nature* 233, 551 (1971).
253. MAURER, H. K. (Papierwerke Waldhof-Aschaffenburg A.G.): Stable antileukemic 5'-(adamantanecarbonyl)-N⁶-(3-methyl-2-butenyl)-adenosine. *Ger. Offen.* 2,112 (Cl. C 07 d), 14. Sep. 1972, Appl. P 21 12 263.3, 13. Mar. 1971. *Chem. Abstr.* 78, 4491 u (1973).
254. MELBY, L. R., and D. R. STROBACH: Oligonucleotide syntheses on insoluble polymer supports. I. Stepwise synthesis of trithymidine diphosphate. *J. Amer. Chem. Soc.* 89, 450 (1967).
255. — — — Oligonucleotide syntheses on insoluble polymer supports. II. Pentathymidine tetraphosphate. *J. Org. Chem.* 34, 421 (1969).
256. — — — Oligonucleotide syntheses on insoluble polymer supports. III. Fifteen di(deoxy-ribonucleoside)monophosphates and several trinucleoside diphosphates. *J. Org. Chem.* 34, 427 (1969).
257. MERRIFIELD, R. B.: Automated synthesis of peptides. *Science* 150, 178 (1968).
258. MICHELSON, A.: The chemistry of nucleosides and nucleotides. London-New York: Academic Press. 1963.
259. MICHNIWICZ, J. J., O. S. BHANOT, J. GOODCHILD, S. K. DIER, R. H. WIGHTMAN, and S. A. NARANG: Benzoylated DEAE-Sephadex. Its preparation and application. *Biochim. Biophys. Acta* 224, 626 (1970).
260. MICHNIWICZ, J. J., C. P. BAIL, K. ITAKURA, N. KATAGIRI, and S. A. NARANG: Fractionation of synthetic deoxyribopolynucleotides on silica-gel-thin-layer plates. *J. Chromatog.* 85, 159 (1973).
261. MILLER, P. S., K. N. FANG, N. S. KONDO, and P. O. P. TS'O: Syntheses and properties of adenine and thymine nucleoside alkyl phosphotriesters, the neutral analogs of dinucleoside monophosphates. *J. Amer. Chem. Soc.* 93, 6657 (1971).
262. MITSUMO, Y. (Takeda Chem. Industries, Ltd.): Adenosine-2'(3'), 5'-diphosphate. *Jap. Pat.* 7000,870 (Cl. 16 E 611.2), 12. Jan. 1970, Appl. 8. Feb. 1967. *Chem. Abstr.* 72, 111788 u (1970).
263. MITSUMORI, O., K. KATO, and J. KIMURA: Selective phosphorylation of the 5'-hydroxy groups of thymidine and uridine. *J. Amer. Chem. Soc.* 91, 6510 (1969).
264. MITSUMORI, O., J. KIMURA, and Y. FUJISAWA: Studies on nucleosides and nucleotides. II. Selective acylation of 5'-hydroxyl group of thymidine. *Bull. Chem. Soc. Japan* 45, 245 (1972).
265. MIURA, K., and T. UEDA: A convenient synthesis of diribonucleoside monophosphates by the use of unblocked nucleosides. *Chem. Pharm. Bull. (Tokyo)* 19, 2567 (1971).
266. MIURA, K., M. SHIGA, and T. UEDA: Nucleosides and Nucleotides. VI. Preparation of diribonucleoside monophosphates containing 4-thiouridine. *J. Biochem.* 73, 1279 (1973).
267. MIYAUCHI, K., Y. MATSUMOTO, T. FURUYA, and K. UCHIDA: Microbial phosphorylation of inosine and guanosine. *Jap. Pat.* 70 35,236 (Cl. C 12 d), 11. Nov. 1970, Appl. 31. Mar. 1966 (Yamasa Shoyu Co. Ltd.). *Chem. Abstr.* 74, 63175 j (1971).
268. MIZUNO, Y., T. ITOH, and H. TAGAWA: New acetylating agents for nucleosides N-acetyl cyclohydroxamic acids. *Chem. and Ind.* 1498 (1965).
269. MIZUNO, Y., and T. SASAKI: The synthesis of dinucleoside phosphates of natural linkages by the anhydronucleoside method. *Tetrahedron Letters* 4579 (1965).
270. MIZUNO, Y., T. SASAKI, T. KAWAI, and H. IGARASHI: Nucleotides I. The reaction of cyclouridines with benzyl hydrogen phosphoric benzoic anhydride. *J. Org. Chemistry* 30, 1533 (1965).
271. MIZUNO, Y., W. LUMM, K. TSUCHIDA, and K. IKEDA: Novel protecting group for the synthesis of 7a-D-pentofuranosyl-hypoxanthines. *J. Org. Chemistry* 37, 39 (1972).
272. MOHR, S. C., and R. E. THACH: Application of ribonuclease T₁ to the synthesis of oligonucleotides of defined base sequence. *J. Biol. Chem.* 244, 6566 (1969).
273. MONFARLER, R. L., and G. A. FISCHER: Mammalian deoxynucleoside kinases. I. Deoxycytidine kinase. Purification, properties and kinetic studies with cytosine arabinoside. *J. Biol. Chem.* 243, 4298 (1968).
274. MOON, M. W., and H. G. KHORANA: Studies on Polynucleotides. LV. The use of mesitylchloride in the synthesis of internucleotide bonds. *J. Amer. Chem. Soc.* 88, 1805 (1966).
275. MORAVEK, J.: Formation of oligonucleotides during heating of a mixture of uridine 2',3'-phosphate and uridine. *Tetrahedron Letters* 1707 (1967).
276. MORAVEK, J., J. KOPECKY, and J. SKODA: Thermal phosphorylations IV. Formal. of a natural internucleotide bond in oligonucleotides formed by heating uridine-2',3'-phosphate with uridine. *Coll. Czech. Chem. Comm.* 33, 960 (1968).
277. — — — Thermal phosphorylations V. Fractionation of products of thermal reaction of uridylic acid with uridine using gel filtration and ion exchange chromatography. *Coll. Czech. Chem. Comm.* 33, 4407 (1968).
278. — — — Thermal phosphorylations VI. Formation of oligonucleotides from uridine-2',3'-monophosphate. *Coll. Czech. Chem. Comm.* 33, 4120 (1968).
279. MORGAN, A. R.: Studies on polynucleotides. XCIV. Transcription of DNA's with repeating nucleotide sequences. *J. Mol. Biol.* 52, 441 (1970).
280. MUKAIYAMA, T., and M. HASHIMOTO: Phosphorylation of alcohols and phosphates by oxidation-reduction condensation. *Bull. Chem. Soc. Jap.* 44, 106 (1971).
281. MUSHIKA, Y., T. HATA, and T. MUKAIYAMA: New phosphorylating reagent. III. Preparation of mixed diesters of phosphoric acid by the use of an activatable protecting group. *Bull. Chem. Soc. Japan* 44, 232 (1971).
282. MUSHIKA, Y., and N. YONEDA: New phosphorylating reagent. IV. Preparation of the mixed phosphoric diesters of dl- α -tocopherol and ethylene glycol analogs by means of 2-chloromethyl-4-nitrophenyl phosphorodichloridate. *Chem. Pharm. Bull. (Tokyo)* 19, 687 (1971).
- 282a. MYLES, A., W. HUTZENLAUB, G. REITZ, and W. PFEIDERER: Nucleotide I. Synthese und Eigenschaften von Thymidyl-(3'→3''), (3'→5'')- und (5'→5'')-thymidin; in preparation.
283. NAGYVARY, J., and J. S. ROTR: Studies on the synthesis of the natural internucleotide bond by the use of cyclonucleosides. *Tetrahedron Letters* 617 (1965).
284. NAKAYAMA, K., and H. TANAKA: Production of nucleic acid related substances. XXXVIII. Production of uridine 5'-monophosphate and orotidine 5'-mono-phosphate by *Brevibacterium ammoniagenes*. *Agr. Biol. Chem.* 35, 518 (1971).
285. NARA, T., T. KOMURO, M. MISAWA, and S. KINOSHITA: Production of nucleic acid related substances by fermentative processes. XXIX. Growth responses of *Brevibacterium ammoniagenes*. *Agr. Biol. Chem.* 33, 1030 (1969).
286. NARANG, S. A., T. M. JACOB, and H. G. KHORANA: Studies on polynucleotides. LXIII. Deoxyribopolynucleotides containing repeating trinucleotide sequences. The polymerization of protected deoxyribonucleotides. *J. Amer. Chem. Soc.* 89, 2167 (1967).
287. NARANG, S. A., K. DHEER, and J. J. MICUNIEWICZ: A new general method for the synthesis of deoxyribopolynucleotides bearing a 5'-phosphomonoester end group. *J. Amer. Chem. Soc.* 90, 2702 (1968).

289. NARANG, S. A., and S. K. DHEER: Chemical synthesis of three deoxyribododecanucleotide chains of defined sequence. *Biochemistry* 8, 3443 (1969).
289. NARANG, S. A., O. S. BHANOT, J. GOODCHILD, and R. WIGHTMAN: Use of substituted phenol as phosphate protecting group in the synthesis of deoxyribo-oligo-nucleotides bearing 5'-phosphomonoester end group. *J. Chem. Soc. D Chem. Commun.* 91 (1970).
290. NARANG, S. A., O. S. BHANOT, J. GOODCHILD, J. MICINIOWICZ, R. A. WIGHTMAN, and S. K. DHEER: Use of new protecting groups in the synthesis of deoxyribo-oligo-nucleotides of defined sequence. *J. Chem. Soc. D Chem. Commun.* 516 (1970).
- 290a. NARANG, S. A., O. S. BHANOT, J. GOODCHILD, R. H. WIGHTMAN, and S. K. DHEER: A new general method for the synthesis of phosphate-protected deoxyribo-oligo-nucleotides. *IV. J. Amer. Chem. Soc.* 94, 6183 (1972).
291. NARANG, S. A., K. TRAKURA, C. P. BAHU, and Y. Y. WIGNER: Chemical synthesis of two deoxyribo-oligonucleotide fragments containing the natural sequence of T4 lysozyme gene. *Biochem. Biophys. Res. Commun.* 49, 445 (1972).
292. NARANG, S. A., K. TRAKURA, and R. H. WIGHTMAN: A simplification in the synthesis of deoxyribo-oligonucleotides. *Can. J. Chem.* 50, 769 (1972).
293. NARANG, S. A., and J. J. MICINIOWICZ: Thin-layer chromatography of synthetic polynucleotides. *Part III. Anal. Biochem.* 49, 379 (1972).
294. NAVLOR, R., and P. T. GILMAN: Studies on Some Interactions and Reactions of Oligonucleotides in Aqueous Solution. *Biochem. J.* 5, 2722 (1966).
295. NEDRAL, V. K., N. I. SOKOLOVA, Z. A. SHABAROVA, and M. A. PROKOF'EV: Chemical matrix synthesis of oligonucleotides in aqueous solutions. *Dokl. Akad. Nauk SSSR* 205, English translation p. 1114 (1972).
296. NELSON, T.: A novel chemical synthesis for oligoribonucleotides. *Chem. Commun.* 1139 (1969).
297. NELSON, T., and E. S. WERSTUJK: Oligoribonucleotide synthesis. II. Preparation of 2'-O-tetrahydropyran derivatives of adenosine and cytidine necessary for insertion in stepwise synthesis. *Can. J. Chem.* 49, 493 (1971).
298. — — — Oligoribonucleotide synthesis III. Synthesis of trinucleotides using a stepwise phosphotriester method. *Can. J. Chem.* 49, 3004 (1971).
299. NELSON, T., E. V. WASTROWSKI, and E. S. WERSTUJK: Oligoribonucleotide synthesis. V. Preparation of 2'-O-tetrahydropyran derivatives of guanosine and their insertion into a general stepwise synthesis. *Can. J. Chem.* 51, 1068 (1973).
300. NELSON, T., and E. S. WERSTUJK: Synthesis of the anticodon loop of *E. coli* methionine transfer ribonucleic acid. *J. Amer. Chem. Soc.* 96, 2295 (1974).
301. NEJEDLY, Z., H. SKODOVA, K. HYAS, and J. SKODA: New possibilities of enzyme synthesis of radioactive nucleotides. II. Phosphoribosylation of radioactive bases of nucleic acids by the catalytic effect of unpurified cell-free extract of *Brevibacterium ammoniagenes*. *J. Label. Compounds* 6, 3 (1970).
302. NEUMAN, M. W., W. F. NEUMAN, and K. LANE: Possible role of crystals in the origins of life. IV. The phosphorylation of nucleotides. *Curr. Mod. Biol.* 3, 277 (1970).
303. NIKOLENKO, L. N., V. N. NEZAVIAT'KO, and M. N. SEMENOVA: Selective N-benzoylation of cytidine 5'-monophosphate. *Zh. Obshch. Khim.* 39, 223 (1969).
304. OGILVIE, K. K., and R. L. LETSINGER: Use of isobutyloxycarbonyl as a blocking group in preparation of 3'-O-*p*-monomethoxytritylimidine. *J. Org. Chemistry* 34, 2465 (1967).
305. OGILVIE, K. K., and D. IWACHIA: Nucleotide syntheses using O², 2-anhydrouridine. *Can. J. Chem.* 48, 862 (1970).
306. OGILVIE, K. K., and K. KROEGER: Synthesis of oligothymidylates on an insoluble polymer support. *Can. J. Chem.* 50, 1211 (1972).
307. OGILVIE, K. K.: The tert-butylidimethylsilyl group as a protecting group in deoxynucleosides. *Can. J. Chem.* 51, 3799 (1973).
308. OHTSUKA, E., M. W. MOON, and H. G. KIORANA: The synthesis of deoxyribopolynucleotides containing repeating dinucleotide sequences. *J. Amer. Chem. Soc.* 87, 2954 (1965).
309. OHTSUKA, E., K. MURAO, M. UHASAWA, and M. IKEYAMA: A new method for the synthesis of protected ribooligonucleotides with 3'-phosphate end groups. *J. Amer. Chem. Soc.* 91, 1537 (1969).
310. — — — Studies on transfer ribonucleic acids and related compounds. I. Synthesis of ribooligonucleotides using aromatic phosphoramidates as a protecting group. *J. Amer. Chem. Soc.* 92, 3441 (1970).
311. OHTSUKA, E., M. UHASAWA, and M. IKEYAMA: Studies on transfer ribonucleic acids and related compounds. II. A method for synthesis of protected ribooligonucleotides using a ribonuclease. *J. Amer. Chem. Soc.* 92, 3445 (1970).
312. — — — Polynucleotides. VIII. A new method for the synthesis of protected deoxyribooligonucleotides with 5'-phosphate. *J. Amer. Chem. Soc.* 92, 5507 (1970).
313. — — — Studies on transfer ribonucleic acids and related compounds. III. Synthesis of hexanucleotide having the sequence of the yeast alanine transfer ribonucleic acid 3' end. *J. Amer. Chem. Soc.* 93, 2296 (1971).
314. OHTSUKA, E., H. TAGAWA, and M. IKEYAMA: Studies on r-RNA's and related compounds. IV. A simple method for the synthesis of ribotrinucleotides. *Chem. Pharm. Bull.* 19, 139 (1971).
315. OHTSUKA, E., A. KUMAR, and H. G. KIORANA: Studies on Polynucleotides. CVII. Total synthesis of the structural gene for an alanine transfer ribonucleic acid from yeast. Synthesis of a dodecadeoxynucleotide and a hexadeoxynucleotide corresponding to the nucleotide sequences I to 12. *J. Mol. Biol.* 72, 309 (1972).
316. OHTSUKA, E., S. MORIOKA, and M. IKEYAMA: Formation of phosphodiester linkages by oxidation of a phosphoramidate. *Tetrahedron Letters* 2353 (1972).
317. — — — Studies on transfer ribonucleic acids and related compounds. V. Synthesis of ribonucleotides with phosphomonoester end groups on a polymer support. *J. Amer. Chem. Soc.* 94, 3229 (1972).
318. OHTSUKA, E., M. UHASAWA, S. MORIOKA, and M. IKEYAMA: Studies on Transfer Ribonucleic Acids and Related Compounds. VI. Synthesis of Yeast Alanine Transfer Ribonucleic Acid 3'-terminal Nonanucleotides and 5'-terminal Hexanucleotides. *J. Amer. Chem. Soc.* 95, 4725 (1973).
319. OHTSUKA, E.: Chemical synthesis of oligo- and poly-nucleotides. In: *Methoden der Organischen Chemie* (Houben-Weyl, ed.), in press.
320. OKAZAKI, R., and A. KORNBERG: Deoxythymidine kinase of *E. coli* I. Purification and some properties of the enzyme. *J. Biol. Chem.* 239, 269 (1964).
321. — — — Deoxythymidine kinase of *E. coli* II. Kinetics and feedback control. *J. Biol. Chem.* 239, 275 (1964).
322. McOMIE, J. F. W.: Protective groups. *Advances Org. Chemistry* 3, 191 (1963).
323. ÖSTERBERG, R., L. E. ORGEL, and R. LÖRRMANN: Further studies of urea-catalyzed phosphorylation reactions. *J. Mol. Evol.* 2, 231 (1973).
324. OTT, D. G., V. N. KERR, E. HANSRURY, and F. N. HAYES: Chemical synthesis of nucleoside triphosphates. *Anal. Biochem.* 21, 469 (1967).
325. OUCHI, S., T. SOWA, K. TSUNODA, and S. SENOJO (Asahi Chem. Industry Co., Ltd.): Direct phosphorylation of nucleosides. *Jap. Pat.* 7016,708 (Cl. 16 E 461), 10. Jun. 1970, Appl. 14. Jul. 1966. *Chem. Abstr.* 73, 66866a (1970).
326. OUCHI, S., T. SOWA, S. KATO, T. OSAWA, and S. SENOJO (Asahi Chem. Industry Co., Ltd.): 5-Phosphorylation of unprotected nucleosides. *Jap. Pat.* 7108,854 (Cl. C 07 d), 5. Mar. 1971, Appl. 20. Jan. 1967. *Chem. Abstr.* 75, 36571 z (1971).
327. PACE, N. R., D. H. L. BISHOP, and S. SPIEGELMAN: The Immediate Precursor of Viral RNA in the Q_β-Replicase Reaction. *Proc. Natl. Acad. Sci. US* 59, 139 (1968).

328. PAETKAU, V. H., and H. G. KHORANA: Preparation of a circular bihelical deoxyribonucleic acid containing repeating dinucleotide sequences. *Biochemistry* 10, 1511 (1971).
329. PÄIVINEN, E., and N. S. TIKKIMIROVA-SIDOROVA: Selective 4-N-acetylation of 2'-deoxycytidine 5'-phosphate. *Zh. Obshch. Khim.* 41, 2076 (1971).
330. PÄIVINEN, E., E. N. MONOZOVA, and N. S. TIKKIMIROVA-SIDOROVA: Acetylation of dinucleotides and synthesis of trinucleotides. *Zh. Obshch. Khim.* 41, 219 (1971).
331. PARKS, R. E., JR., and R. P. AGARWAL: Nucleotide kinases. In: *The Enzymes*. VI. (P. D. BOYER, ed.), 3rd edition. New York-London: Academic Press, 1972.
332. PHILIPP, M., and H. SELIGER: Formylated deoxynucleotidyl triphosphates as potential substrates of deoxynucleotide polymerizing enzymes. Abstracts. 164th ACS-meeting. New York. CARB 5 (1972).
333. ———: Unpublished results.
334. POCHON, F., M. LENG, and A. M. MICHELSON: Photochimie des polynucleotides. III. Étude de la luminescence de poly-nucléotides à température ordinaire. *Biochim. Biophys. Acta* 169, 350 (1968).
335. PUCHON, F., and A. M. MICHELSON: Polynucleotide analogues. XIV. Poly N²-dimethyl-guanilate. *Biochim. Biophys. Acta* 182, 17 (1969).
336. PONDOR, S. K., and I. TINOCO JR.: Enzymatic synthesis of oligoguanilic acids containing 2'-5' phosphodiester linkages. *Biochem. Biophys. Res. Commun.* 34, 569 (1969).
337. PONDOR, S. K.: Synthetic action of ribonuclease T₁. *Biochim. Biophys. Acta* 209, 455 (1970).
338. ———: On self-interacting oligoribonucleotides. I. absorption and optical rotatory dispersion of 2'-5' and 3'-5'-oligoguanilic acids. *Biochemistry* 10, 2415 (1971).
339. PONGS, O., and P. O. P. TS'Ö: Polymerization of 5'-deoxyribonucleotides with β -imidazole-4(5)-propanoic acid. *Biochem. Biophys. Res. Commun.* 36, 475 (1969).
340. ———: Polymerization of unprotected 2'-deoxyribonucleoside-5'-phosphates at elevated temperature. *J. Amer. Chem. Soc.* 93, 5241 (1971).
341. POONIAN, M. S., E. F. NOWOSWIAT, and A. L. NUSSBAUM: Nucleoside-S-alkyl phosphorothates. VII. A fragment from the nonsense strand of a modified S-peptide "gene". *J. Amer. Chem. Soc.* 94, 3992 (1972).
342. POTAROV, V. K., O. G. CHERKINAKHIEVA, Z. A. SHABAROVA, and M. A. PROKOF'EV: Synthesis of oligonucleotides on polymer carriers. Synthesis of deoxy-[thimidyl(3'→5')-adenyl(3'→5')-adenyl(3'→5')-adenosine]. *Dokl. Akad. Nauk SSSR* 196, 360 (1971).
343. POTAROV, V. K., S. I. TURKIN, and Z. A. SHABAROVA: Application of Sephadex LH-20 in the synthesis of oligonucleotides on polymeric carriers. *Zh. Obshch. Khim.* 42, 2349 (1972).
344. RABINOWITZ, J.: 265. Recherche sur la formation et la transformation des esters. LXXIII. (I). Reaction de condensation et/ou de phosphorylation, en solution aqueuse, de divers composés organiques à fonction —OH, —COOH, —NH₂ ou autre à l'aide de polyphosphates linéaires ou cycliques. *Helv. Chim. Acta* 52, 2663 (1969).
345. RABINOWITZ, J., S. CHANG, and C. PONNAMPERUMA: Phosphorylation by way of inorganic phosphate as a potential prebiotic process. *Nature* 218, 442 (1968).
346. RAJAPALEE, F. J. M.: A convenient synthesis of 2',3',5'-tri-O-acetyladenosine and -uridine. *Angew. Chem.* 10, 75 (1971).
347. RAMEL, A., E. HEIMER, S. ROY, and A. L. NUSSBAUM: Gel filtration of acylated oligonucleotides. *Anal. Biochem.* 41, 323 (1971).
348. RANDERATHI, K., and E. RANDERATHI: Thin-layer Separation Methods for Nucleic Acid Derivatives. In: S. P. COLOWICK and N. O. KAPLAN, *Methods in Enzymology*, Vol. XII (L. GROSSMANN and K. MOLDAVE, eds.), p. 323. New York-London: Academic Press, 1967.
349. RATLIFF, R. L., and F. N. HAYES: Enzymatic synthesis of a three-section block co-polymer of (thymidylate, deoxyguanylate and deoxyadenylate. *Biochim. Biophys. Acta* 134, 203 (1967).
350. REESE, C. B., and R. SAFFHILL: Oligonucleotide synthesis via phosphotriester intermediates: The phenyl protecting group. *J. Chem. Soc. D, Chem. Commun.* 767 (1968).
351. REESE, C. B., and J. C. M. STEWART: Methoxyacetyl as a protecting group in ribonucleoside chemistry. *Tetrahedron Letters* 4723 (1968).
352. REESE, C. B.: A systematic approach to oligoribonucleotide synthesis. *Chim. Organ. du Phosphore (Colloqu. Intern. du centre national de la recherche scientifique, eds.)* 182, 319 (1969).
353. REESE, C. B., J. H. VAN BOOM, G. R. OWEN, J. PRASTON, and T. RAYINDRANATHAN: Synthesis of oligoribonucleotides. IX. Preparation of ribonucleoside 2'-acetyl 5'-esters. *J. Chem. Soc. C* 3320 (1971).
354. REICHEL, W., E. STENGEL, and H. SELIGER: Kinetik der Schutzgruppenabspaltung an Nucleosiden mittels ¹H-NMR-Spektroskopie. *Chem. Ber.* 107, 611 (1974).
355. RENZ, M., R. LOURMANN, and L. E. ORGEL: Catalysts for the polymerization of adenosine cyclic 2',3'-phosphate on a poly (U) template. *Biochim. Biophys. Acta* 240, 463 (1971).
356. RICHARDS, G. M., D. J. TUTAS, W. J. WÉCITER, and M. LASKOWSKI SR.: Hydrolysis of dinucleoside monophosphates containing arabinose in various internucleotide linkages by exonuclease from the venom of crotalus adamanteus. *Biochemistry* 6, 2908 (1967).
357. RICHARSON, C. C.: Phosphorylation of nucleic acid by an enzyme from T4-bacteriophage infected *Escherichia coli*. *Proc. Natl. Acad. Sci. US* 54, 158 (1965).
358. ROKOS, H., W. HUTZENLAUN, A. MYLES, and W. FLEDERER: Nucleotide, II: Isomerisierung der Internucleotidbindung bei der Abspaltung der Phosphor-Schutzgruppe von Phosphorsäureestern. In preparation.
359. RÖSSNER, E.: Synthese von Oligodeoxyribonucleotiden mit 3'-terminalem Ribonucleotid durch Cooligokondensation. Diplomarbeit, Univ. Freiburg, 1972.
360. ROYCHOUNURY, R., and H. KÖSSEL: Synthetic polynucleotides. Enzymic synthesis of ribonucleotide terminated oligodeoxynucleotides and their use as primers for the enzymic synthesis of polydeoxynucleotides. *Eur. J. Biochem.* 22, 310 (1971).
361. ROYCHOUNURY, R.: Enzymic Synthesis of Polynucleotides. Oligodeoxynucleotides with one 3'-terminal Ribonucleotide as Primers for Polydeoxynucleotide Synthesis. *J. Biol. Chem.* 247, 3910 (1972).
- 361a. ROYCHOUNURY, R., S. KÜHN, H. SCHOTT, and H. KÖSSEL: Enzymic polynucleotide synthesis primed by polyvinylalcohol linked oligothymidylate. *FEBS Letters* 50, 140 (1975).
362. RUBINSTEIN, M., and A. PATCHORNIK: Polymers as chemical reagents. Use of poly-3,5-diethylstyrene sulfonyl chloride for the synthesis of internucleotide bonds. *Tetrahedron Letters* 2281 (1972).
363. SACHDEV, H. S., and N. A. STARKOVSKY: Enzymatic removal of acyl protecting group: The use of dihydrocinamoyl group in oligonucleotide synthesis and its cleavage α -chymotrypsin. *Tetrahedron Letters* 733 (1969).
364. SAFFHILL, R.: Selective phosphorylation of the cis-2',3'-diol of unprotected ribonucleosides with trimetaphosphate in aqueous solution. *J. Org. Chemistry* 35, 2881 (1970).
365. SATTO, M., Y. FURUCHI, K. TAKEISHI, M. YOSHIDA, M. YAMASAKI, K. ARIMA, H. HAYATSU, and T. UKITA: Synthesis of diribonucleoside monophosphates by use of a nonspecific ribonuclease from *Bacillus subtilis*. *Biochim. Biophys. Acta* 195, 299 (1969).
366. SANGER, F., J. E. DONELSON, A. R. COULSON, H. KÖSSEL, and D. FISCHER: Use of DNA polymerase I primed by a synthetic oligonucleotide to determine a nucleotide sequence in phase I DNA. *Proc. Natl. Acad. Sci. US* 70, 1209 (1973).
367. SANNO, Y., and A. NOHARA: Phosphorylation of 2',3'-isopropylideneinosine by heating or ultraviolet irradiation in the presence of phosphoric acid and nitriles. *Chem. Pharm. Bull. (Tokyo)* 16, 2056 (1968).

- 367a. SANO, H., and G. FEIX: Ribonucleic acid ligase activity of DNA ligase from *T. infected E. coli*. *Biochemistry* 13, 5110 (1974).
- 367b. SCHEFFLER, I. E., and C. C. RICHARDSON: Chemical and enzymatic studies of DNA covalently linked to ficoll. *J. Biol. Chem.* 247, 5736 (1972).
368. SCHERT, K. H.: Untersuchungen an poly-5-Hydroxymethyluridylsäure und poly-5-Methyluridylsäure. *Biochim. Biophys. Acta* 134, 17 (1967).
369. — Die Benzylester von Desoxydinucleosidphosphaten und 5'-O-(β , β , β -trichloräthylphosphoryl)-thymidyl-3'-5'-thymidyl-3'-5'-thymidin. *Tetrahedron Letters* 3243 (1967).
370. — Über die Synthese und Eigenschaften von 4-Thiouridyl-3'-5'-4-thiouridin, 4-Thiouridyl-3'-5'-uridin und Uridyl-3'-5'-4-thiouridin. *Biochim. Biophys. Acta* 166, 285 (1968).
371. SCHERT, K. H., and E. GAERTNER: Die Polymerisation von 4-Thiouridin-5'-diphosphat und 4-Thiouridin-5'-diphosphat durch Polynucleotidphosphorylase aus *Micrococcus lysodeikticus*. *Biochim. Biophys. Acta* 182, 1 (1969).
372. SCHERT, K. H.: Enzymatic polymerization of 5-methyl-4-thiouridine-5'-diphosphate by polynucleotide phosphorylase from *Escherichia coli*. *Biochim. Biophys. Acta* 209, 445 (1970).
373. SCHERT, K.-H., and P. FAERNER: Synthesis and properties of poly(s²C), a new Poly(C) analog. *Eur. J. Biochem.* 24, 385 (1971).
374. SCHERTERS, H., H. G. GASSEN, and H. MATTHAEI: Codon-anticodon interaction studied with oligonucleotides containing 3-deazauridine, 4-deoxyuridine or 3-deaza-4-deoxyuridine. I. Synthesis by primer-dependent polynucleotide phosphorylase of oligonucleotides containing modified nucleosides. *Biochim. Biophys. Acta* 272, 549 (1972).
375. SCHNEIDER-BENLOEHR, H., R. LOHRMANN, J. SULSTON, B. J. WEIMANN, L. E. ORCEL, and H. TODD MILES: Non-enzymic synthesis of deoxyadenylate oligonucleotides on a polyuridylic template. *J. Mol. Biol.* 37, 151 (1968).
376. SCHNEIDER-BENLOEHR, H., R. LOHRMANN, J. SULSTON, L. E. ORCEL, and H. TODD MILES: Specificity of template-directed synthesis with adenine nucleosides. *J. Mol. Biol.* 47, 257 (1970).
377. SCHOTT, H.: New dihydroxyboryl-substituted polymers for column-chromatographic separation of ribonucleoside-deoxyribonucleoside mixtures. *Angew. Chem.* 11, 824 (1972).
378. — Polyvinyl alcohol substituted by nucleotides as carrier for liquid phase oligonucleotide synthesis. *Angew. Chem.* 12, 246 (1973).
379. SCHOTT, H., D. FISCHER, and H. KÖSSEL: Synthesis of four undecanucleotides complementary to a region of the coat protein cistron of phage fd. *Biochemistry* 12, 3447 (1973).
380. SCHOTT, H., and H. KÖSSEL: Synthesis of phage specific deoxyribonucleic acid fragments. I. Synthesis of four undecanucleotides complementary to a mutated region of the coat protein cistron of fd phage deoxyribonucleic acid. *J. Amer. Chem. Soc.* 95, 3778 (1973).
381. SCHOTT, H., E. RUDLOFF, P. SCHMIDT, R. ROYCHOUDHURY, and H. KÖSSEL: A dihydroxyboryl-substituted methacrylic polymer for the column chromatographic separation of mononucleotides, oligonucleotides, and transfer ribonucleic acid. *Biochemistry* 12, 932 (1973).
382. SCHOTT, H., F. BRANDSTETTER, and E. BAYER: Liquid-Phase Synthese von Oligo-thymidylphosphaten. *Makromol. Chem.* 173, 247 (1974).
383. SCHOTT, H.: Chemische Synthese eines phagenspezifischen DNA-Fragments. *Makromol. Chem.* 175, 1683 (1974).
384. SCHWARTZ, A. W., and F. N. HAYES: Synthesis of a polydeoxyribonucleotide containing an internal pyrophosphate linkage. *Biochim. Biophys. Acta* 138, 604 (1967).
385. SCHWARTZ, A. W.: Specific phosphorylation of the 2'- and 3'-position in ribonucleosides. *J. Chem. Soc. D, Chem. Comm.* 1393 (1969).
386. SCHWARTZ, A., and C. PONNAMPERUMA: Phosphorylation of adenosine with linear polyphosphate salts in aqueous solution. *Nature* 218, 449 (1968).
- 386a. SEDELNIKOVA, E. A., and S. M. ZHENODAROVA: Stepwise synthesis of oligonucleotides. I. 5-O-(α -alkoxyalkyl) derivatives of uridine 3'-phosphate. *Zh. Obshch. Khim.* 38, 2234 (1968).
387. SEDELNIKOVA, E. A., O. A. SMOLYANINOVA, and S. M. ZHENODAROVA: Stepwise synthesis of oligonucleotides. III. Structural analysis of α -alkoxyalkyl groups in O-(α -alkoxyalkyl) derivatives of nucleosides and nucleotides. *Zh. Obshch. Khim.* 38, 2245 (1968).
388. SEKIYA, T., Y. FUNUCHI, M. YOSHIDA, and T. UKITA: Ribonuclease-T₁ catalyzed Synthesis of Triribonucleoside Diphosphates having a Guanosine Residue at the 5'-End. *J. Biochem. (Tokyo)* 63, 514 (1968).
389. SELIGER, H.: Versuche zur Einführung und Reaktivierung von Enolgruppen als Schutzgruppen an Phosphorsäuren. Diplom thesis. Technische Hochschule Darmstadt. 1963.
390. SELIGER, H., and F. CRAMER: Nucleophile Substitutionen mit Pyrimidinnucleosid-natriumsalzen. *Angew. Chem.* 81, 577 (1969). *Angew. Chem. Internat. Edit.* 8, 609 (1969).
391. SELIGER, H.: Chlorameisensäureester von Nucleosiden — neue Zwischenprodukte für Synthesen mit Nucleinsäurebausteinen. *Tetrahedron Letters* 4043 (1972).
392. SELIGER, H., and G. AUMANN: Oligonucleotide synthesis on a polymer support soluble in water and pyridine. *Tetrahedron Letters* 2911 (1973).
393. SELIGER, H.: Ein neuer Weg zur Synthese von Polystyrol und Styrol-Copolymeren mit primären aromatischen Aminogruppen. *Makromol. Chem.* 169, 83 (1973).
394. SELIGER, H., G. AUMANN, V. GENRICH, M. PHILIPP, and E. RÖSSNER: Improvements in the development of rational methods for oligonucleotide synthesis. Abstracts. XXIVth Internat. Congr. of pure and applied chemistry, Hamburg, p. 136 (1973).
395. SELIGER, H., G. AUMANN, and R. L. LETSINGER: Progress in the synthesis of oligonucleotides on polymer supports. Contributed Papers, Internat. Symposium on Macromolecules, Rio de Janeiro. 221 (1974).
396. SELIGER, H., G. AUMANN, V. GENRICH, M. PHILIPP, E. RÖSSNER, and H. SCHÜTZ: Sequence-specific cooligocondensation of nucleic acid constituents — a new approach to polynucleotide synthesis. Contributed Papers, Internat. Symposium on Macromolecules, Rio de Janeiro. 222 (1974).
- 396a. SELIGER, H., and G. AUMANN: Oligonucleotidsynthese an unvernetzten Copolymeren des Vinylalkohols und N-Vinylpyrrolidons. *Makromol. Chem.*, in press.
397. SELIGER, H.: Polymers in aid of polynucleotide chemistry. Current topics in chemistry manuscript in preparation.
- 397a. SELIGER, H.: Handelsübliche Polymerale Träger in der Oligonucleotidsynthese. I. these eines Pentanucleosidtetraphosphats an Merckogel OR 1 000 000st. *Makromol. Chem.*, in press.
398. SELIGER, H., H. SCHÜTZ, E. SAUR, and M. PHILIPP: Oligonucleotide synthesis with nucleotide-3'-formyl esters. *J. Carbohydr. Nucleosides, Nucleotides*, in press.
- 398a. SELIGER, H., E. RÖSSNER, G. AUMANN, V. GENRICH, M. HOLMREK, T. KNÄBLE, and M. PHILIPP: Sequenzspezifische Cooligocondensation von Nucleinsäurebausteinen mit Affinitätschutzgruppen. I. Desoxyoligonucleotide mit Ribouridin-Terminus. *Makromol. Chem.*, in press.
- 398b. SELIGER, H., H. SCHÜTZ, and M. PHILIPP: Sequenzspezifische Cooligocondensation von Nucleinsäurebausteinen mit Affinitätschutzgruppen. II. Cooligomere von 5'-O-(p -Methoxytrityl)-thymidin und Desoxynucleotiden. *Makromol. Chem.*, in press.

399. SGARAMELLA, V., J. H. VAN DE SANDE, and H. G. KIORANA: Studies on polynucleotides. C. A novel joining reaction catalyzed by the T₄-polynucleotide ligase. *Proc. Nat. Acad. Sci. U.S.A.* 67, 1468 (1970).
400. SGARAMELLA, V., and H. G. KIORANA: Studies on Polynucleotides. CXII. Total synthesis of the structural gene for an alanine transfer RNA from yeast. Enzymic joining of the chemically synthesized polydeoxyribonucleotides to form the DNA duplex representing nucleotide sequence 1 to 20. *J. Mol. Biol.* 72, 427 (1972).
401. — Studies on polynucleotides. CXVI. A further study of the T₄ ligase-catalyzed joining of DNA at base-paired ends. *J. Mol. Biol.* 72, 493 (1972).
402. SGARAMELLA, V., K. KLEPPE, T. TERAU, N. K. GUPTA, and H. G. KIORANA: Studies on Polynucleotides. CXIII. Total synthesis of the structural gene for an alanine transfer RNA from yeast. Enzymic joining of the chemically synthesized segments to form the DNA duplex corresponding to nucleotide sequence 17 to 30. *J. Mol. Biol.* 72, 445 (1972).
403. SHABAROVA, Z. A., and M. A. PROKOF'EV: A model of enzymatic synthesis of the internucleotide bond between oligodeoxyribonucleotides. *FEBS Letters* 11, 237 (1970).
404. SHENYAKIN, M. M., YU. A. OVCHINNIKOV, A. A. KURYUSIKIN, and I. V. KOZHEVNIKOVA: Synthesis of peptides in solution on a polymeric support. I. Synthesis of glycyl-L-leucyl-glycine. *Tetrahedron Letters* 2323 (1965).
405. SIEN, T.-Y., and K. H. BOSWELL (Merck and Co., Inc.): 5'-Adamantoyl-2'-deoxy-5-(methylamino)-uridine. *US Pat.* 3,676,422 (Cl. 260/214.5R; C 07d). 11. Jul. 1972. *Appl.* 73,206, 17. Sep. 1970. *Chem. Abstr.* 77, 114817s (1972).
406. SHIMIZU, B., M. ASAI, and T. NISHIMURA: Synthetic nucleotides. I. A convenient synthesis of ribonucleotides. *Chem. Pharm. Bull. (Tokyo)* 15, 1847 (1967).
407. SHIMIZU, T., and R. L. LETSINGER: Synthesis of deoxyguanylyldeoxyguanosine on an insoluble polymer support. *J. Org. Chem.* 33, 708 (1968).
408. — Hydrolysis of p-nitrophenyl(deoxyguanylyl-deoxyguanosine succinate) by deoxyguanylyldeoxyguanosine N-acetylhistidine on polycytidylic acid. *Bull. Chem. Soc. Japan* 44, 584 (1971).
409. — The preparation of deoxyguanosine oligomers on an insoluble polymer support. *Bull. Chem. Soc. Japan* 44, 1673 (1971).
410. — Hydrolyses of p-Nitrophenyl(oligodeoxyribonucleotide succinate)s by oligodeoxyribonucleotide N-acetylhistidates on polycytidylic acid. *Bull. Chem. Soc. Japan* 46, 3270 (1973).
411. SHINSKII, N. G., N. N. PRAEDBRASHENSKAYA, M. G. IVANOVSKAYA, Z. A. SHABAROVA, and M. A. PROKOF'EV: Phosphorylation of nucleosides by pyrophosphoryl chloride. *Dokl. Akad. Nauk SSSR* 184, 622 (1969).
412. SMITH, J., K. H. SCHIET, and E. M. GOTTSCHALK: The enzymatic synthesis of poly 4-thiouridylic acid by polynucleotide phosphorylase from *Escherichia coli*. *Biochim. Biophys. Acta* 204, 371 (1970).
413. SMITH, J., P. STREINKE, U. NIEBHALLA, H. VORBRÜGGEN, and K. H. SCHIET: 2'-O-methylcytidine 5'-diphosphate as substrate for polynucleotide phosphorylase from *Escherichia coli*. *Biochim. Biophys. Acta* 228, 654 (1971).
414. SMIRNOV, V. D., M. G. IVANOVSKAYA, E. V. IL'INA, Z. A. SHABAROVA, and M. A. PROKOF'EV: Synthesis of the phenylalanine amide of a pentadeoxynucleotide. *Dokl. Akad. Nauk SSSR* 206, English translation p. 1133 (1972).
415. SMIT, J., and J. CATLIN: Abnormal course of phosphorylation with methyl phosphate. *Tetrahedron Letters* 5081 (1970).
416. SMIT, J., and F. CRAMER: Oligonucleotide compounds. XXXVI. Synthesis of uridylyl-(5'-3')-uridylyl-(5'-5')-uridylyl-(3'-5')-uridine and its priming activity for polynucleotide phosphorylase. *Collect. Czech. Chem. Commun.* 35, 1456 (1970).
417. SMIT, J.: Protection of the internucleotide bond after its synthesis. An approach to the synthesis of oligonucleotide chains. *Tetrahedron Letters* 3437 (1972).
418. SMIT, J.: Oligonucleotide compounds. XXXIX. Triester synthesis of oligonucleotides in the ribo series. *Collect. Czech. Chem. Commun.* 37, 846 (1972).
419. — Oligonucleotide compounds. XL. Aspects of the triester synthesis in the ribo series. *Collect. Czech. Chem. Commun.* 37, 1870 (1972).
420. — Oligonucleotide compounds. XLI. On the reaction of ribonucleoside 2'(3')-phosphates with dimethylformamide dimethylacetal. *Collect. Czech. Chem. Commun.* 37, 4088 (1972).
421. — A remark on the preparation of protected guanosine-3'-phosphate by means of a mixture of ribonucleases T₁ and T₂. *Collect. Czech. Chem. Commun.* 39, 969 (1974).
422. — Combined synthesis of oligonucleotides in the deoxy series. *Collect. Czech. Chem. Commun.* 39, 972 (1974).
423. SOMMER, H., and F. CRAMER: Synthese von Oligodeoxyribonucleotiden mit 5'-terminaler Phosphatgruppe. *Angew. Chem.* 84, 710 (1972).
424. SOWA, T., S. OUCHI, and T. OSAWA (Asahi Chem. Industry Co., Ltd.): 5'-Nucleotides by a direct phosphorylation. *Jap. Pat.* 71 02,025 (Cl. C 07d), 19. Jan. 1971. *Appl.* 16. Dec. 1966. *Chem. Abstr.* 74, 112405v (1971).
425. SOWA, T., K. SATO, S. OUCHI, T. OSAWA, and S. SEN (Asahi Chem. Industry Co., Ltd.): Selective phosphorylation to give nucleotides. *Jap. Pat.* 71 04,986 (Cl. C 07d), 6. Feb. 1971. *Appl.* 7. Jan. 1967. *Chem. Abstr.* 75, 36578g (1971).
426. SPORN, M. B., D. M. BERKOWITZ, R. P. GLINSKI, A. B. ASH, and C. L. STEVENS: Irreversible inhibition of nuclear exoribonuclease by thymidine-3'-fluorophosphate and p-haloacetamidophenyl nucleotides. *Science* 164, 1408 (1969).
427. SRIVASTAVA, P. C., and M. M. DHAR: The use of a purine cyclonucleoside for the synthesis of a dinucleotide phosphate. *Tetrahedron Letters* 47 (1968).
428. SRIVASTAVA, P. C., K. L. NAGPAL, and M. M. DHAR: The synthesis of a natural dinucleoside phosphate derivative with the aid of a purine cyclonucleoside. *Experientia* 24, 657 (1968).
429. — Synthesis of dinucleoside phosphates by reaction of 5'-chloro-5'-deoxynucleosides with nucleotide anions. *Experientia* 25, 356 (1969).
- 429a. STUART, A., and H. G. KIORANA: Studies on polynucleotides. XXXIII. The labelling of end groups in polynucleotide chains: The selective acetylation of terminal hydroxyl groups in deoxyribopolynucleotides. *J. Biol. Chem.* 239, 3885 (1964).
430. SULSTON, J., R. LOHRMANN, L. E. ORGEL, and H. TODD MILES: Nonenzymatic synthesis of oligoadenylates on a polycytidylic acid template. *Proc. Nat. Acad. Sci. (U.S.)* 59, 726 (1968).
431. — — — Specificity of oligonucleotide synthesis directed by polynucleic acid. *Proc. Nat. Acad. Sci. (U.S.)* 60, 409 (1968).
432. SULSTON, J., R. LOHRMANN, L. E. ORGEL, H. SCHNEIDER-BERNLOEHR, B. J. WEIMAN, and H. TODD MILES: Non-enzymic oligonucleotide synthesis on a polycytidylic template. *J. Mol. Biol.* 40, 227 (1969).
433. SUSSMAN, J. L., I. BARZILAY, M. KEREN-ZUR, and Y. LAPIDOT: Correlation of the differences in conformation between 2'-5' and 3'-5' dinucleoside monophosphates with their behaviour on a Sephadex LH-20 column. *Biochim. Biophys. Acta* 308, 189 (1973).
434. SVETKOWSKI, M., and D. SHUGAR: Poly 5-ethyluridylic acid, a polynucleic acid analogue. *J. Mol. Biol.* 47, 57 (1970).
435. TAJIMA, K., and T. HATA: Simple protecting group protection-purification handle for polynucleotide synthesis. II. *Bull. Chem. Soc. Japan* 45, 2608 (1972).
436. TAUNTON-RICHY, A., Y.-H. KIM, C. J. CROSSCUP, and N. A. STARKOVSKY: Oligonucleotide synthesis. II. The use of substituted trityl groups. *J. Org. Chem.* 37, 956 (1972).

437. TAUNTON-RIGBY, A.: Oligonucleotide synthesis. III. Enzymatically removable acyl protecting groups. *J. Org. Chem.* 38, 977 (1973).
438. TAZAWA, I., S. TAZAWA, L. M. STENPEL, and P. O. P. TS'O: L-adenyl-(3'-5')-L-adenosine and L-adenyl-(2'-5')-L-adenosine. *Biochemistry* 9, 3499 (1970).
439. TIANG, M. N., and M. GRUNBERG-MANAGO: Enzymatic synthesis of polyguanylic acid and copolymers containing guanylic acid. In: *Methods in enzymology*, XII B, 522 (L. GROSSMAN and K. MOLDAVE, eds.). New York-London: Academic Press, 1968.
440. TIGERSTROM, R. V., and M. M. SMITH: Oligodeoxyribonucleotides: Chemical synthesis in anhydrous base. *Science* 167, 1266 (1970).
441. TIKHOMIROVA-SIDOROVA, N. S., E. M. KOGAN, V. A. SYSOEV, and G. E. USTYUZHANIN: Alcoholysis of pyrimidine nucleoside 2',3'-cyclophosphates with purine nucleosides in frozen solutions in presence of pancreatic ribonuclease. *Zh. Obshch. Khim.* 41, 2570 (1971).
442. TIKHOMIROVA-SIDOROVA, N. S., G. E. USTYUZHANIN, T. N. KALACHEVA, and V. I. KALUGINA: Hydrolytic and synthetic activity of guanylic ribonuclease of actinomycetes in frozen solutions. *Zh. Obshch. Khim.* 41, 2108 (1971).
- 442a. TORRENCE, P. F., J. A. WATERS, and B. WITKOP: Unexpected conformational stability of poly(2'-azido-2'-deoxyuridylic acid). *J. Amer. Chem. Soc.* 94, 3638 (1972).
443. TORRENCE, P. F., and B. WITKOP: Enzymatic synthesis of polynucleotides containing 5,6-methylene- and 5,6-dihydroxyuridines. *Biochemistry* 11, 1737 (1972).
444. TORRENCE, P. F., A. M. BORST, J. A. WATERS, and B. WITKOP: Synthesis and characterization of potential interferon inducers. Poly(2'-azido-2'-deoxyuridylic acid). *Biochemistry* 12, 3962 (1973).
445. TORRENCE, P. F., J. A. WATERS, C. E. BUCKLER, and B. WITKOP: Effect of pyrimidine and ribose modifications on the antiviral activity of synthetic polynucleotides. *Biochem. Biophys. Res. Commun.* 52, 890 (1973).
446. TSAPALIS, C. M., and S. A. NARANG: On the fidelity of phage T₄-induced polynucleotide ligase in the joining of chemically synthesized deoxyribonucleotides. *Biochem. Biophys. Res. Commun.* 39, 631 (1970).
447. TSOU, K. C., and K. F. YU: Synthesis of deoxypolynucleotides on an isotactic polymer support. *J. Macromol. Sci. Chem.* A7(5), 1097 (1973).
448. UCHIC, J. T., M. UCHIC, and A. D. BROOM: Studies on polyribonucleotides. Synthesis of a polynosinic: 6-thiinosinic acid copolymer. *Biochem. Biophys. Res. Commun.* 51, 494 (1973).
449. — — — Studies on polyribonucleotides. Polymerization of 6-chloropurine riboside-5'-diphosphate; personal communication.
450. UCHIDA, T., T. ARIMA, and F. EGAMI: Specificity of RNase U₂. *J. Biochem. (Tokyo)* 67, 91 (1970).
451. UEDA, T., and J. J. FOX: Mononucleotides. *Advan. Carbohydr. Chem. Biochem.* 22, 307 (1967).
452. UEDA, T., and I. KAWAI: A convenient synthesis of ribonucleoside 2',3'-cyclic phosphates from ribonucleosides and ribonucleotides. *Chem. Pharm. Bull. (Tokyo)* 18, 2303 (1970).
453. UESUGI, S., M. YASUNOTO, M. IKEHARA, K. N. FANG, and P. O. P. TS'O: Synthesis and properties of the dinucleoside monophosphate of adenine 8-thiocyclophosphonate. *J. Amer. Chem. Soc.* 94, 5480 (1972).
454. VAN DE SANDE, J. H., M. H. CARUTHERS, V. SCABANELLA, T. YAMADA, and H. G. KHORANA: Studies on Polynucleotides. CXIV. Total synthesis of the structural gene for an alanine transfer RNA from yeast. Enzymic joining of the chemically synthesized segments to form the DNA duplex corresponding to nucleotide sequence 46 to 77. *J. Mol. Biol.* 72, 457 (1972).
455. VERLANDER, M. S., R. LOHRMANN, and L. E. ORGEL: Catalysts for the self-polymerization of adenosine cyclic 2',3'-phosphate. *J. Mol. Evol.* 2, 303 (1973).
456. VERLANDER, M. S., and L. E. ORGEL: Analysis of high molecular weight material from the polymerization of adenosine cyclic 2',3'-phosphate. *J. Mol. Evol.* 3, 115 (1974).
457. WEBER, H., and H. G. KHORANA: Studies on Polynucleotides. CIV. Total synthesis of the structural gene for an alanine transfer ribonucleic acid from yeast. Chemical synthesis of an isodeoxyribonucleotide corresponding to the nucleotide sequence 21 to 40. *J. Mol. Biol.* 72, 219 (1972).
458. WEIMANN, G., and H. G. KHORANA: Studies on Polynucleotides. XVII. On the mechanism of internucleotide bond synthesis by the carboximide method. *J. Amer. Chem. Soc.* 84, 4329 (1962).
459. WEIMANN, B. J., R. LOHRMANN, L. E. ORGEL, H. SCHNEIDER-BERNLOEHR, and J. E. SULSTON: Template-directed synthesis with adenosine-5'-phosphorimidazole. *Science* 161, 387 (1968).
460. WEITH, H. L., J. L. WIENERS, and P. T. GUHAM: Synthesis of cellulose derivatives containing the dihydroxyboryl group and a study of their capacity to form specific complexes with sugars and nucleic acid components. *Biochem.* 9, 4396 (1970).
461. WELLS, R. D., and R. M. WARTLE: Review of synthesis of specific DNA polymer. *Biochem. Mol. Biol. (ed. K. BURTON)*. Butterworth and Medical and Techn. Publishing Co. (1973).
462. WERSTIUK, E. S., and T. NELSON: Oligoribonucleotide Synthesis. IV. Approach to block synthesis. *Can. J. Chem.* 50, 1283 (1972).
463. WERSTIUK, E. S., and T. NELSON: Oligoribonucleotide synthesis. VI. Selective deblocking of the 5'-O-triphenylmethoxycetyl grouping in protected dinucleotides. *Can. J. Chem.* 51, 1889 (1973).
464. WESTHEIMER, F. H., C. CLAPP, and J. WISEMAN: Monomeric metaphosphates and metaphosphorimidates. XXIVth IUPAC Congress of Pure and Applied Chemistry. Hamburg, 1973. Abstracts of Papers, p. 410.
465. WIGHTMAN, R. H., S. A. NARANG, and K. ITAKURA: A novel phosphate protecting group for oligonucleotide synthesis. *Can. J. Chem.* 50, 456 (1972).
466. WINDHOLZ, T. B., and D. B. R. JOHNSTON: Trichloroethoxycarbonyl: a generally applicable protecting group. *Tetrahedron Letters* 2555 (1967).
467. WU, R. C.-P., D. TU, and R. PADMANABHAN: Nucleotide sequence analysis of DNA. XII. The chemical synthesis and sequence analysis of a dodecadeoxynucleotide which binds to the endolysin gene of bacteriophage lambda. *Biochem. Biophys. Res. Commun.* 55, 1092 (1973).
- 467a. WU, R.: personal communication.
468. WÜNSCH, E.: Synthese von Peptidnaturstoffen. Problematik des leutigen Forschungsstandes. *Angew. Chem.* 83, 773 (1971).
469. YAMASHITA, T., and T. KATO (Ajinomoto Co. Inc.): 2',3'-O-Substituted nucleoside-5'-phosphates. *Jap. Pat.* 69 27,979 (Cl. 16 E 611.2), 19. Nov. 1969, Appl. 3. Dec. 1971. *Chem. Abstr.* 72, 21913b (1970).
470. YIR, K. F., and K. C. TSOU: A new polymer-support method for the synthesis of ribooligonucleotide. *J. Amer. Chem. Soc.* 93, 3272 (1971).
471. YONEI, S., A. KUNIMAKA, and H. YOSHINO (Yamasa Shoyu Co., Ltd.): *Jap. Pat.* 71 31,865 (Cl. C 07 d), 17. Sep. 1971, Appl. 7. Feb. 1969. *Chem. Abstr.* 75, 152051 d (1971).
472. YOSHIKAWA, M., T. KATO, and T. TAKENISHI: Selective phosphorylation of unprotected nucleosides. *Bull. Chem. Soc. Jap.* 42, 3505 (1969).
473. YOSHUKAWA, M., M. SAKURABA, and K. KUSASAKI: Phosphorylation. IV. Phosphorylation of nucleosides with phosphorus trihalide. *Bull. Chem. Soc. Jap.* 43, 456 (1970).
474. YURKEVICH, A. M., L. S. VARSIAVSKAYA, and I. I. KOLODKINA: Reaction of nucleosides with diphenylboric acid esters. *Zh. Obshch. Khim.* 38, 2115 (1968).
475. YURKEVICH, A. M., I. I. KOLODKINA, L. S. VARSIAVSKAYA, V. I. BORODULINA-SHETZ,

476. J. P. RUDAKOVA, and N. A. PREOBRAZHENSKI: The reaction of phenylboronic acid with nucleosides and mononucleosides. *Tetrahedron* 25, 477 (1969).
477. ZARYTOVA, V. F., V. K. POTAPOV, Z. A. SHABAROVA, and D. G. KNORRE: Synthesis of oligonucleotides on polymer supports. Synthesis of oligodeoxynucleotides containing deoxyguanylic acid. *Dokl. Akad. Nauk SSSR* 199, 1072 (1970).
478. ZEMLUCKA, J., J. BERANEK, and J. SMRT: Preparation and methanolysis of uridine, 6-azauridine and 6-aza-cytidine-O-formyl derivatives. *Coll. Czech. Chem. Comm.* 27, 2784 (1962).
479. ZEMLUCKA, J., and J. SMRT: The reaction of O², 5'-cyclophosphoridate and O², 5'-cyclophosphoridate derivatives with nucleotides — A new approach to the synthesis of the 3'-5'-internucleotidic bond. *Tetrahedron Letters* 2681 (1964).
480. ZEMLUCKA, J.: 2'-3'-O-(ethoxymethylene)uridine. Synthetic procedures in nucleic acid chemistry. 1, 422. New York: Wiley, 1968.
481. ZEMLUCKA, J., and S. CHLADEK: New method of dephosphorylation of ribonucleoside-2'(3')-phosphates. *Tetrahedron Letters* 715 (1969).
482. ZEMLUCKA, J.: Nucleic acid components and their analogues. CXXXII. Alkylation of some nucleic acid components and their analogues with dimethylformamide acetals. *Collect. Czech. Chem. Commun.* 35, 3572 (1970).
483. — Acetalation and acetylation of pyrimidine nucleosides in dioxane-acetonitrile-hydrogen chloride. *J. Org. Chem.* 36, 2383 (1971).
484. ZEMLUCKA, J., and S. CHLADEK: Synthesis of 2'(3')-O-ethyl derivatives of cytidyl-(3'-5')-inosine and 2'-deoxycytidyl-(3'-5')-adenosine. *Biochemistry* 10, 1521 (1971).
485. ZEMLUCKA, J., and J. P. HORVITZ: Nucleosides. XIII. The concurrent introduction of two different blocking groups into some ribonucleosides. *J. Org. Chem.* 36, 2809 (1971).
486. ZHENODAROVA, S. M., and M. I. HARABOVA: The enzymic synthesis of adenylyl-(3'-5')-cytidine. *Biochim. Biophys. Acta* 169, 559 (1968).
487. ZMUDZKA, B., and D. SHUGAR: Role of the 2'-hydroxyl in polynucleotide conformation: poly 2'-O-methyluridylic acid. *Acta Biochim. Polon.* 18, 321 (1971).
488. ZMUDZKA, B., M. TICHY, and D. SHUGAR: The structure of poly 2'-O-methylcytidylic acid and its complexes with polyinosinic acid. *Acta Biochim. Polon.* 19, 149 (1972).
489. ZWIERZAK, A., and R. GRAMZE: Organophosphorus esters II. Novel approach to the synthesis of S-alkyl phosphorothioates. *Z. Naturforsch.* 26b, 386 (1971).
490. ZWIERZAK, A., and M. KLUNA: Organophosphorus esters — I. *t*-butyl as protecting group in phosphorylation via nucleophilic displacement. *Tetrahedron Letters* 3163 (1971).

(Received March 1, 1974)

Namenverzeichnis. Author Index

Kursiv gedruckte Seitenzahlen beziehen sich auf Literaturverzeichnisse

Page numbers printed in *italics* refer to References

- | | | | |
|--------------------|--------------------|-------------------|--------------------|
| Ackman, R. G. | 46 | Anderson, J. W. | 291 |
| Ackmann, R. J. | 46 | Anderson, W. A. | 135, 164 |
| Aberlath, E. | 107 | Ando, K. | 176 |
| Abelson, D. H. | 43 | Ando, M. | 249 |
| Aberhardt, D. J. | 249 | Andresen, B. | 144 |
| Abraham, R. J. | 116 | Andresen, D. D. | 48 |
| Abushanab, E. | 258 | Anet, F. A. L. | 133, 160, 164, 166 |
| Achenbach, H. | 249 | Angermann, N. S. | 164 |
| Adam, G. | 249, 255, 263 | Angier, R. B. | 174 |
| Adams, J. Q. | 163 | Anirudhan, C. A. | 249 |
| Adinarayana, D. | 249 | Antonov, V. K. | 110 |
| Agadzhanyan, Z. E. | 110 | Aebi, H. | 117 |
| Agarwal, K. L. | 351, 483, 485, 492 | Aoka, Y. | 118 |
| Agarwal, R. P. | 500 | Aota, K. | 255 |
| Aguliar-Santos, G. | 249 | Aoyagi, R. | 264 |
| Agvada, V. | 249, 254 | Aplini, R. T. | 249 |
| Almad, M. | 490 | Appelqvist, L.-A. | 283, 291 |
| Almann, G. | 256 | Appleby, R. S. | 291 |
| Ajil, S. J. | 118 | ApSimon, J. W. | 148, 163, 165 |
| Akers, H. A. | 109 | Arakawa, H. | 249 |
| Akutsu, H. | 483 | Argoudelis, A. D. | 117 |
| Alais, J. | 249 | Arich, G. | 44, 49 |
| Albonico, S. M. | 163, 257 | Arima, K. | 116, 501 |
| Albrecht, P. | 45, 47, 49 | Arima, T. | 506 |
| Ali, M. S. | 114 | Arison, B. H. | 109, 117 |
| Allard, M. | 249 | Arnold, R. R. | 163, 262 |
| Allen, C. F. | 291 | Arthur, H. R. | 249 |
| Allen, C. M. | 75, 112 | Asada, Y. | 260 |
| Allen, F. H. | 249 | Asai, M. | 483, 504 |
| Allehand, A. | 165, 166 | Asaka, Y. | 249 |
| Alvarez, C. | 261 | Asao, T. | 249 |
| Alves, H. M. | 258 | Asao, Y. | 117 |
| Amagaeva, A. A. | 483 | Ash, A. B. | 488, 505 |
| Amil, R. G. | 116 | Atkins, C. L. | 109 |
| Anderson, G. D. | 249 | Audisio, G. | 253 |
| Anderson, J. S. | 291 | August, J. T. | 487 |

Form PTO-1449 U.S. Department of Commerce

(REV. 8-83) Patent and Trademark Office

INFORMATION DISCLOSURE CITATION
(use several sheets if necessary)Atty. Docket No.
ENZ-5(D6)(C2)

Serial No. 08/479,997

Applicants: Dean L. Engelhardt et al.

Filed: June 7, 1995

Group: 1656

U.S. PATENT DOCUMENTS

EXAMINER INITIAL	DOCUMENT NUMBER	DATE	NAME	CLASS	SUB CLASS	FILING DATE IF APPRO- PRIATE

FOREIGN PATENT DOCUMENTS

DOCUMENT NUMBER	DATE	COUNTRY	CLASS	SUB CLASS	TRAN- SLATION YES NO

OTHER DOCUMENTS (Including Author, Title, Date, Pertinent Pages, Etc.)

	Pollack, S. E. and Auld, D. S., "Fluorescent Nucleotide Triphosphate Substrates for Snake Venom and Phosphodiesterase," <u>Analytical Biochemistry</u> 127: 81-88 (1982)
	Scopes, D. I. C., et al., "Defined Dimensional Changes in Enzyme Cofactors: Fluorescent "Stretched-Out" Analogs of Adenine Nucleotides," <u>Science</u> 195: 296-298 (1977)
	Onodera, M. and Yagi, K., "Synthesis of 2-(Dansylamino)Ethyl Triphosphate and its Properties as a Fluorescent Substrate of Heavy Meromyosin-ATPase," <u>Biochimica Et Biophysica</u> 253: 254-265 (1971)
	Yarbrough, L. R., "Synthesis and Properties of a New Fluorescent Analog of ATP: Adenosine-5'-Triphospho-γ-1-(5-Sulfonic Acid) Naphthylamidate," <u>Biochemical and Biophysical Research Communications</u> 81(1): 35-41 (1978)
	Silver, M. S. and Fersht, A. R., "Direct Observation of Complexes Formed between recA Protein and a Fluorescent Single-Stranded Deoxyribonucleic Acid Derivative," <u>Biochemistry</u> 21: 6066-6072 (Received April 16, 1982)

EXAMINER

DATE CONSIDERED

*EXAMINER: Initial if citation considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

Form PTO-1449 U.S. Department of Commerce (REV. 8-83) Patent and Trademark Office INFORMATION DISCLOSURE CITATION (use several sheets if necessary)	Atty. Docket No. ENZ-5(D6)(C2)	Serial No. 08/479,997
	Applicants: Dean L. Engelhardt et al.	
	Filed: June 7, 1995	Group: 1656

U.S. PATENT DOCUMENTS

EXAMINER INITIAL	DOCUMENT NUMBER	DATE	NAME	CLASS	SUB CLASS	FILING DATE IF APPRO- PRIATE

FOREIGN PATENT DOCUMENTS

DOCUMENT NUMBER	DATE	COUNTRY	CLASS	SUB CLASS	TRAN- SLATION YES NO

OTHER DOCUMENTS (Including Author, Title, Date, Pertinent Pages, Etc.)

	Crane, L. J. and Miller, D. L., "Guanosine Triphosphate and Guanosine Diphosphate as Conformation-Determining Molecules. Differential Interaction of a Fluorescent Probe with the Guanosine Nucleotide Complexes of Bacterial Elongation Factor Tu," <u>Biochemistry</u> 13(5): 933-938 (1974)
	Hertz, H. S. and Zachau, H. G., "Kinetic Properties of Phenylalanyl-tRNA and Seryl-tRNA Synthetases for Normal Substrates and Fluorescent Analogs," <u>Eur. Journal of Biochemistry</u> 37: 203-213 (1973)
	Wu, F. Y. H. and Wu, C. W., "Fluorescent Affinity Labeling of Initiation Site on Ribonucleic Acid Polymerase of <i>Escherichia coli</i> ," <u>Biochemistry</u> 13(12): 2562-2566 (1974)
	McKenzie, R. L., et al., "Fluorescent Conjugates of Natural and Biosynthetic Polynucleotides," <u>Biochimica Et Biophysica ACTA</u> 277: 306-322 (1972)
	Tournon, J., "Fluorescence Probing of Nucleic Acids: I. singly and doubly labeled dithymidine phosphate: fluorescence and energy transfer studies," <u>Nucleic Acids Research</u> 2(8): 1261-1273 (1975)
	Erlanger, B. F., et al., "Use of Antibodies to Nucleosides and Nucleotides in Studies of Nucleic Acids in Cells," <u>Methods in Enzymology</u> 60: 302-307 (1975)
EXAMINER	DATE CONSIDERED
*EXAMINER: Initial if citation considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.	

Form PTO-1449 U.S. Department of Commerce (REV. 8-83) Patent and Trademark Office INFORMATION DISCLOSURE CITATION (use several sheets if necessary)	Atty. Docket No. ENZ-5(D6)(C2)	Serial No. 08/479,997
	Applicants: Dean L. Engelhardt et al.	
	Filed: June 7, 1995	Group: 1656

U.S. PATENT DOCUMENTS

EXAMINER INITIAL	DOCUMENT NUMBER	DATE	NAME	CLASS	SUB CLASS	FILING DATE IF APPRO- PRIATE

FOREIGN PATENT DOCUMENTS

DOCUMENT NUMBER	DATE	COUNTRY	CLASS	SUB CLASS	TRAN- SLATION YES NO

OTHER DOCUMENTS (Including Author, Title, Date, Pertinent Pages, Etc.)

	Yarbrough, L. R., et al., "Synthesis and Properties of Fluorescent Nucleotide Substrates for DNA-dependent RNA Polymerases," <u>The Journal of Biological Chemistry</u> 254(23): 12069-12073 (1979)
	Wintermeyer, W. and Zachau, H. G., "Fluorescent Derivatives of Yeast tRNA ^{Phe} ," <u>Eur. Journal of Biochem</u> 98: 465-475 (1979)
	Reines, S. A. and Schulman, L. H., "A New Method for Attachment of Fluorescent Probes to tRNA," <u>Methods in Enzymology</u> 61: 146-157 (1979)
	Barrio, J. R., et al., "Synthesis of Modified Nucleoside 3', 5'-Bisphosphates and Their Incorporation into Oligoribonucleotides with T4 RNA Ligase," <u>Biochemistry</u> 17(11): 2077-2081 (1978)
	Koenig, P., et al., "Pyrene Derivatives as Fluorescent Probes of Conformation Near the 3' Termini of Polyribonucleotides," <u>Biopolymers</u> 16: 2231-2242 (1977)
	Menzel, H. M., "On the Phe-tRNA induced binding of fluorescent oligonucleotides to the ribosomal decoding site," <u>Nucleic Acids Research</u> 4(8): 2881-2892 (1977)
	Leonard, N. J. and Tolman, G. L., "Fluorescent Nucleosides and Nucleotides," <u>Annals of the New York Academy of Sciences</u> 255: 43-58 (1975)
	Gavrilovskaya, I. N., et al., "Immunofluorescent Demonstration of Double-Stranded RNA and Virus Antigen in RNA Virus-Infected Cells," <u>Virology</u> 62: 276-279 (1974)

EXAMINER

DATE CONSIDERED

*EXAMINER: Initial if citation considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

Form PTO-1449 U.S. Department of Commerce (REV. 8-83) Patent and Trademark Office INFORMATION DISCLOSURE CITATION (use several sheets if necessary)	Atty. Docket No. ENZ-5(D6)(C2)	Serial No. 08/479,997
	Applicants: Dean L. Engelhardt et al.	
	Filed: June 7, 1995	Group: 1656

U.S. PATENT DOCUMENTS

EXAMINER INITIAL	DOCUMENT NUMBER	DATE	NAME	CLASS	SUB CLASS	FILING DATE IF APPRO- PRIATE

FOREIGN PATENT DOCUMENTS

DOCUMENT NUMBER	DATE	COUNTRY	CLASS	SUB CLASS	TRAN- SLATION YES NO

OTHER DOCUMENTS (Including Author, Title, Date, Pertinent Pages, Etc.)

	Kukhanova, M. K., et al., "Peptidyl-tRNA With A Fluorescent Label: Ribosome Substrates in Peptide Bond Formation," <u>Molecular Biology Reports</u> 1: 397-400 (1974)
	Lynch, D. C. and Schimmel, P. R., "Effects of Abnormal Base Ionizations on Mg ²⁺ Binding to Transfer Ribonucleic Acid as Studied by a Fluorescent Probe," <u>Biochemistry</u> 13(9): 1852-1861 (1974)
	Bergstrom, D. E. and Leonard N. J., "Structure of the Borohydride Reduction Product of Photolinked 4-Thiouracil and Cytosine. Fluorescent Probe of Transfer Ribonucleic Acid Tertiary Structure," <u>Journal of the American Chemical Society</u> 94(17): 6178-6182 (1972)
	Mundry, K. W. and Priess, H., "A Quantitative Technique for Mapping Oligonucleotides on Thin Layers of Cellulose," <u>Biochimica Et Biophysica ACTA</u> 269: 225-236 (1972)
	Munniger, K. O. and Chang, S. H., "A Fluorescent Nucleoside from Glutamic Acid tRNA of <i>Escherichia coli</i> K12," <u>Biochemical and Biophysical Research Communications</u> 46(5): 1837-1842 (1972)
	Erlanger, B. F., et al., "Nucleic Acid-Reactive Antibodies Specific for Nucleosides and Nucleotides," <u>Acta endocrinologica</u> 71: 206-221 (1972).
	Yoshikami, D. and Keller, E. B., "Chemical Modification of the Fluorescent Base in Phenylalanine Transfer Ribonucleic Acid," <u>Biochemistry</u> 10(15): 2969-2976 (1971)
	Rudkin G. T. and Siollar, B. D., "High Resolution Detection of DNA-RNA Hybrids <i>in situ</i> by Indirect Immunofluorescence," <u>Nature</u> 265: 472-473 (1977)
EXAMINER	DATE CONSIDERED

*EXAMINER: Initial if citation considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

Form PTO-1449 U.S. Department of Commerce (REV. 8-83) Patent and Trademark Office INFORMATION DISCLOSURE CITATION (use several sheets if necessary)	Atty. Docket No. ENZ-5(D6)(C2)	Serial No. 08/479,997
	Applicants: Dean L. Engelhardt et al.	
	Filed: June 7, 1995	Group: 1656

U.S. PATENT DOCUMENTS

EXAMINER INITIAL	DOCUMENT NUMBER	DATE	NAME	CLASS	SUB CLASS	FILING DATE IF APPRO- PRIATE

FOREIGN PATENT DOCUMENTS

DOCUMENT NUMBER	DATE	COUNTRY	CLASS	SUB CLASS	TRAN- SLATION YES NO

OTHER DOCUMENTS (Including Author, Title, Date, Pertinent Pages, Etc.)

	Rozovskaya, T. A., et al., "Introduction of a Fluorescent Label at the 3' -OH End of DNA and the 3' -OH End of the Growing RNA Chain," <u>Molekulyama Biologiya</u> 11(3): 598-610 (1977)
	Kossel, H. and Seliger, H., "Recent Advances in Polynucleotide Synthesis," <u>Progress in the Chemistry of Organic Natural Products</u> 32: 297-508 (1975)
EXAMINER	DATE CONSIDERED
*EXAMINER: Initial if citation considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.	